The impact of viruses on the marine deep biosphere

Dissertation

Von der Fakultät für Mathematik und Naturwissenschaften
der Carl-von-Ossietzky Universität Oldenburg zur Erlangung des
Akademischen Grades und Titels eines
Doktors der Naturwissenschaften
Dr. rer. nat.

Angenommene Dissertation
von

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geboren am 14.06.1986 in Neubrandenburg

Oldenburg
2016

Die vorliegende Doktorarbeit wurde in der Zeit von Januar 2012 bis März 2016 am Institut für Chemie und Biologie des Meeres (ICBM) in der Arbeitsgruppe Paläomikrobiologie angefertigt.

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Tag der Disputation: 17.06.2016

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Zusammenfassung

Ziel dieser Arbeit waren Untersuchungen zur der Rolle von Viren auf mikrobielle Lebensgemeinschaften sowie von Viren-Wirt Interaktionen in den extrem oligotrophen Sedimenten des südpazifischen Wirbels. Aufgrund der begrenzt verfügbaren Menge an Proben aus der tiefen Biosphäre, bietet es sich an, Methodenentwicklungen mit anderen Sedimenten voranzustellen. Frühere Studien zeigten, dass die tiefen Sedimente des Wattenmeeres als Modell für die tiefe Biosphäre dienen können.

In dieser Arbeit werden folgend drei Studien an tiefen Sedimenten unterschiedlicher Standorte präsentiert. Die erste Studie befasst sich dabei mit der Analyse von Virenproduktionsraten in Wattsedimenten. Messungen dieser Raten ermöglichen eine Abschätzung der Bedeutung von Viren auf indigene mikrobiellen Gemeinschaften, beispielsweise über die Mortalitätsraten. Hierzu wurden erstmals detaillierte Untersuchungen entlang eines 4 Meter langen Sedimentkerns des Standortes Janssand durchgeführt. Zwei verschiedene Methoden zur Ermittlung von Virenproduktionsraten (i) mittels der Verdünnungsmethode und (ii) über den Einbau von Radio-Isotopen in Virenpartikel, wurden unter anoxischen Bedingungen getestet, letztere davon sogar erstmals in Sedimenten. Im Vergleich zueinander stellte sich die Verdünnungsmethode mit ihren kürzeren Inkubationszeiten von 6 Stunden als sensitiver heraus. Um Veränderungen in den Virenzahlen detektieren zu können, benötigte die Inkorporationsmethode mindestens 4 Tage und teilweise sogar Stimulierung mittels Glukose. Außerdem stellte sich die hierfür erforderliche Ermittlung eines Konversionsfaktors, zur Umwandlung der gemessenen Radioaktivitäten in Virenzahlen, aufgrund von verschiedenen Faktoren als schwierig heraus. Die gefundenen Virenproduktionsraten mittels der Verdünnungsmethode zählten zu den höchsten jemals gemessenen Raten und waren aber dennoch vergleichbar mit früheren Studien. Die berechneten Mortalitätsraten zwischen 6 und 115 % des Bakteriengrundstocks pro Tag lassen den Rückschluss auf einen bedeutenden Einfluss der Viren in diesen Sedimenten zu. Aufgrund diverser Schwierigkeiten, die während dieser Experimente aufgedeckt wurden, entschieden wir uns gegen einen Transfer der Methoden auf die Sedimente des südpazifischen Wirbels.

Die zwei weiteren Studien dieser Arbeit basieren auf Erkenntnissen einer früheren Untersuchung im südpazifischen Wirbel, in der steigende Viren-zu-Bakterien

Verhältnisse mit zunehmender Sedimenttiefe berichtet wurden. Unter Berücksichtigung der extremen Nährstofflimitation der mikrobiellen Gemeinschaften, könnten Virenpartikel (aus Nukleinsäure und Proteinen bestehend) für diese eine leicht abbaubare Nährstoffquelle darstellen. Wir stellten daher die Hypothese auf, dass die indigenen Mikroorganismen die Grundbausteine der Virenpartikel als Kohlenstoffquelle nutzen könnten. Um diese Theorie zu testen, wurden zunächst Messungen für Exoenzym-Aktivitäten sowohl in den Sedimenten als auch anhand von bakteriellen Isolaten aus dem südpazifischen Wirbel durchgeführt. Der erfolgreiche Nachweis von Aminopeptidase-Aktivitäten (zur Spaltung von Proteinbindungen) bewies, dass eine wichtige Grundbedingung zur Nutzung von Virenpartikeln durch mikrobielle Gemeinschaften bereits erfüllt war. Weitere Untersuchungen mit den hungernden bakteriellen Kulturen und Sedimenten des südpazifischen Wirbels und zugefügten Phagen-Konzentraten aus *Rhizobium radiobacter* wurden durchgeführt. Eine Abnahme der Virenzahlen in diesen Kulturen mit der Zeit, unterstützte die ursprüngliche Hypothese.

Summary

The aim of this thesis was to investigate the role of viruses on microbial communities and virus-host interactions in the extremely oligotrophic South Pacific Gyre (SPG) sediments. Due to the in general limited amount of samples from the deep biosphere, establishment of new methods was done in other sediments prior to the main experiments. Former studies demonstrated that subsurface sediments of tidal flats can serve as a model for the deep subseafloor biosphere.

In this thesis, three studies on subsurface sediments of different sites will be presented. The first study reports on pre-experiments for the analysis of viral production rates on tidal-flat sediments. Rate measurements open the possibility to estimate the impact of viruses on indigenous microbial communities, e.g. virus-induced prokaryotic mortality. For this purpose, detailed analyses were performed on a four meter long sediment core derived from the tidal-flat Site Janssand. Two different methods for determining viral production rates (i) the dilution method and (ii) incorporation of labeled ^{3}H-thymidine into virus particles were tested under anoxic conditions. The latter was performed on sediments for the first time. Comparison of both methods revealed that the dilution method was far more sensitive than the radiolabeling method and required short incubation times of six hours, only. For the detection of changes in viral numbers, the radiolabeling method required a minimum of four days and sometimes even glucose-stimulation. Additionally, the establishment of the obligatory factor for the conversion of measured radioactivity into viral production rates turned out to be highly challenging due to several reasons discussed in the study. The viral production rates determined by the dilution method were among the highest rates ever measured, but in a comparable range with other studies. Estimations of virus-induced prokaryotic mortality accounted for 6 to 115 % of the prokaryotic standing stock per day and thus, indicated a moderate impact of the viruses on indigenous microorganisms. Due to several difficulties revealed by the pre-experiments, transfer of neither of the methods was performed on SPG sediments.

The other two studies were based on previous findings that reported virus-to-cell ratios dramatically increased with depth in SPG sediments. Taking into account that indigenous microorganisms face sever nutrient- and energy-limitation, virus particles, composed of mainly nucleic acids and a protein capsule, display easily degradable

organic matter. We hypothesized that microbial communities might potentially feed on the building blocks of viruses. To test this hypothesis, exoenzyme activity measurements were performed on microbial communities of SPG bulk-sediments as well as bacterial isolates of the SPG. The successful detection of aminopeptidase activity confirmed the precondition for substrate uptake by microorganisms. Moreover, feeding experiments with concentrated Rhizobiophages on starving SPG isolates and bulk sediments were conducted. Decreasing viral numbers over time supported our initial hypothesis.

List of publications

The results published in this dissertation have been or will be submitted to international journals:

Preuss F., Engelen B., Kramer S., Engelhardt T, Cypionka H. and Vandieken V. (under revision in Aquat Microb Ecol). *Viral production in an anoxic subsurface sediment of the German Wadden Sea*.

Preuss F., Cypionka H. and Engelen B. (under revision in Front Microbiol). *Microbial communities in deep-subsurface sediments of the South Pacific Gyre exhibit exoenzyme activities*.

Preuss F., Moskwa L.M., Cypionka H. and Engelen B. (to be submitted). *Microbial communities in extremely oligotrophic sediments of the South Pacific Gyre: Do they feed on phages*?

Additional publications not included in this thesis

Dosdall R, Hahn V, **Preuß F**, Kreisel H, Miersch J and Schauer F (2014) "*Characterization of fungi of the genus Mycena isolated from houses thatched with* Phragmites communis *Trin. in Northern Germany: Enzyme pattern and reed decay*". Int Biodeter Biodegr 96: 174-180. doi:10.1016/j.ibiod.2014.09.012

Hofmann K, Kreisel H, Kordon K, **Preuss F**, Kües U and Schauer F (accepted in Mycol Prog) "*The key role of lignin decomposing fungi in the decay of roofs thatched with water reed*"

Presentations at international and national conferences

2016 Association for General and Applied Microbiology (VAAM). Talk: "Microbial communities in extremely oligotrophic sediments of the South Pacific Gyre: Do they eat phages?". Jena, Germany.

2015 Association for General and Applied Microbiology (VAAM). Poster. Marburg, Germany.

2014 ECORD summer school: "Subseafloor Biosphere: Current Advances and Future Challenges". Talk: "Viruses in subsurface sediments". Bremen, Germany

2014 IODP/ ICDP Kolloquium. Poster. Erlangen, Germany

2013 Association for General and Applied Microbiology (VAAM). Poster. Bremen, Germany

2013 IODP/ ICDP Kolloquium. Poster. Freiberg, Germany

2012 IODP Exp. 329 post cruise meeting. Poster. Kona, Hawaii.

2012 IODP/ ICDP Kolloquium. Poster. Kiel, Germany

List of abbreviations

BLAST	Basic local alignment search tool
bp	base pairs
Bq	Becquerel
DFG	German Research Foundation
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dpm	Disintegration per minute
EMBL	European Molecular Biology Laboratory
Exp	Expedition
ICBM	Institute for Chemistry and Biology of the Marine Environment
IODP	International Ocean Discovery Program
LIMS	Laboratory information management system
mbsf	Meter below sea floor
mbsl	Meter below sea level
MCA	7-Amino-4-methylcoumarin
MCA-Leu	L-Leucine-7-amido-4-methylcoumarin
MUF	4-Methylumbelliferone
MUF-P	4-Methylumbelliferyl phosphate
PCR	Polymerase chain reaction
phageFISH	Fluorescence *in-situ* hybridization of phages
q-PCR	Quantitative polymerase chain reaction
RAPD-PCR	Randomly amplified polymorphic deoxyribonucleic acid polymerase chain reaction
Ref	References
rpm	Rounds per minute
SPG	South Pacific Gyre
SRR	Sulfate reduction rates
VCR	Virus-to-cell ratio
VLP	Virus-like particles
WGA	Whole genome amplification

1 Introduction

1.1 The marine subsurface biosphere

The world's oceans and thus, the underlying marine sediments cover around two thirds of the world's surfaces, spanning from the organic-rich continental margins to the oligotrophic open oceans. The vast extension of the oceans makes them the largest interconnected ecosystem on earth (Parkes *et al.*, 1994). So far, living prokaryotes have been detected in almost all investigated sediments (Parkes *et al.*, 1994; Schippers *et al.*, 2005).

1.1.1 Prokaryotic abundance and activity in marine subsurface-sediments

Based on early cell counts, Whitman and colleagues (Whitman *et al.*, 1998) originally estimated that 35-47% of the global biomass is fixed in form of prokaryotes within subsurface sediments and proposed them as "the hidden majority". More recent studies have now shown that these first calculations were overestimated due to the sites chosen for microbial investigations as most studies on the deep-subseafloor biosphere have been performed in close proximity to the continental shelves (D´Hondt *et al.*, 2013). Recalculations after including more oligotrophic oceanic provinces revealed global cell numbers within deep-subseafloor sediments of 2.9×10^{29}, which downsized the initial estimation to ~0.6% of Earth´s total biomass (Kallmeyer *et al*., 2012). Nevertheless, these numbers are still as high as in all ocean waters (1.2×10^{29}), or soils (2.6×10^{29}) (Kallmeyer *et al*., 2012).

Abundance and activity of prokaryotes within sediments are directly linked to the availability of nutrients and electron acceptors (Canfield *et al*., 1993; Jørgensen, 2000; DeLong, 2004). The supply with these substrates depends on organic material being produced within the water column (Azam *et al.*, 1983; Jørgensen, 2000; Parkes *et al*., 2014). Close to the continents, primary production is fueled by nutrients from the land- and sea- site (Azam *et al*., 1983; Poremba *et al*., 1999; van Beusekom and de Jonge, 2002). Accordingly, high amounts of rich organic material reach the seafloor (Gittel *et al*., 2008). Thus, ocean margins and the underlying sediments are characterized by high microbial activities, fast oxygen consumption within the first millimeters to centimeters and steep biochemical gradients (Llobet-Brossa *et al*., 2002; D'Hondt *et al*., 2004; Billerbeck *et al*., 2006; Beck *et al*., 2008). With increasing distance to the continental margins and water depth, the decreasing substrate supply results in

reduced primary production and lower amounts of freshly produced organic material (Figure 1, Jahnke, 1996; Engelen *et al*., 2014). As microbial degradation of the sinking particles already starts during the sedimentation process, the organic matter that reaches the seafloor is also highly recalcitrant (Suess, 1980).

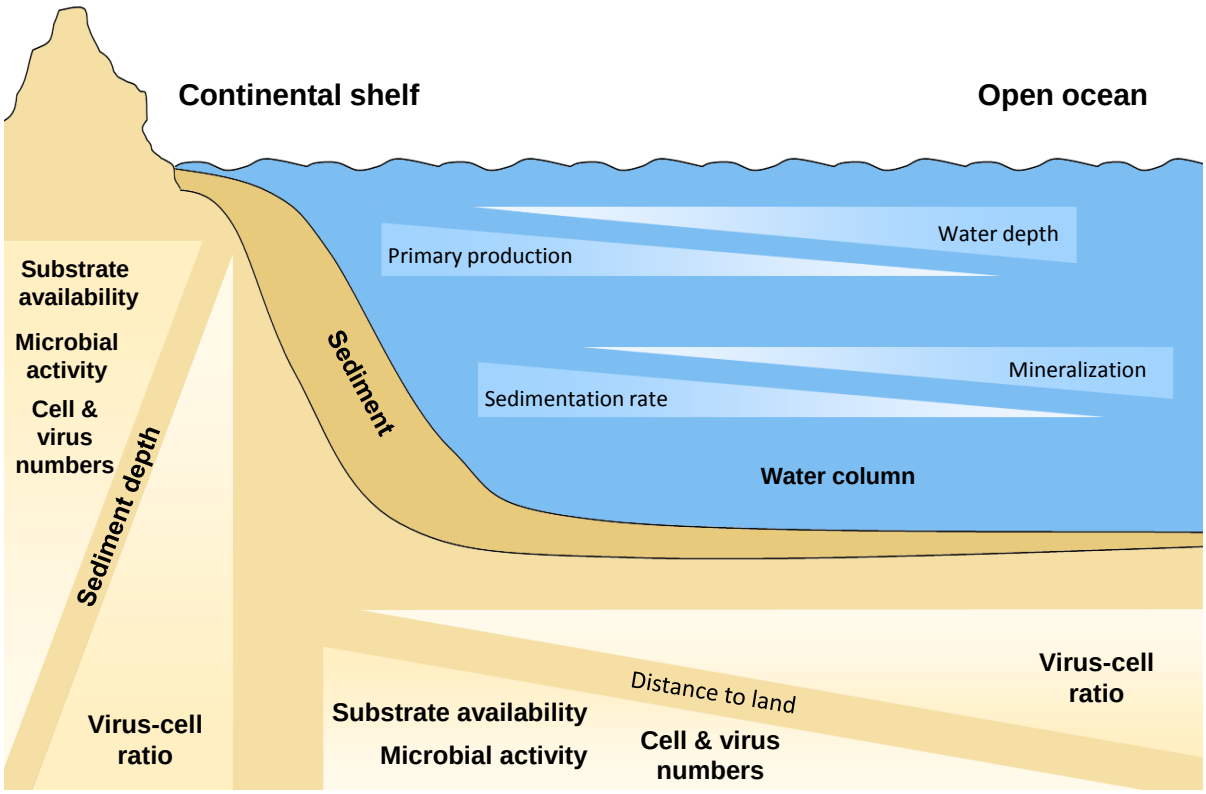

Figure 1: Abundances and activities of cells as wells as virus abundances within marine sediments in relation to substrate availability, primary production, sedimentation rate and sediment depth from the continental margins to the open oceans. Figure taken from (Engelen *et al*., 2014)

1.1.2 Determining the limits of life

Only few decades ago, researchers have agreed that the microbial life would exist only in the first meters below the seafloor (Jørgensen & Boetius, 2007). Since the 1980s this picture has changed due to drilling of deep-subsurface sediments in the frame of the Ocean Drilling Program (ODP). In several studies based on these expeditions, the presence of living microbes in deep sediment layers was investigated (Parkes *et al*., 2000; D'Hondt *et al*., 2004; Schippers *et al*., 2005). Researchers defined the ecosystem one meter below the seafloor as the deep biosphere (Orcutt *et al*., 2013). As a consequence of these findings, the focus of current deep-subsurface research has now changed towards: (i) investigating the extent of the deep biosphere, including the

limits of life and (ii) its impact on the surface world (Hinrichs and Inagaki, 2012; Jørgensen, 2012). Recently, microbial cells have been detected as deep as ~2.5 km below the seafloor, probably the lower border of the subseafloor habitable zone (Inagaki *et al*., 2015). As for adjusting the global cell numbers, extremely oligotrophic marine environments such as the ocean gyres obviously need to be included to study life under extreme energy limitation.

1.1.3 The South Pacific Gyre exhibit the most oligotrophic of marine sediments

Oceans gyres are found north- and southwards of the equator. They are located in large distance to the continental margins and cover around 48 % of the world´s oceans (D'Hondt *et al*., 2009). Being characterized by lowest primary production rates in the photic zone (Jahnke, 1996), this makes them the most oligotrophic regions of the marine realm. Only small amounts of highly recalcitrant organic matter reach the seafloor, resulting in low sedimentation rates and low microbial activity (Røy *et al*., 2012). The South Pacific Gyre (SPG), the largest of the "ocean desserts" with sedimentation rates between 0.008-1.1 mm kyr^{-1} (D'Hondt *et al*., 2009) was target of IODP Expedition 329 in 2010 (D´Hondt *et al*., 2010). The low substrate availability leads to prokaryotic numbers with orders of magnitude lower compared to sediments at ocean margins with equal water depth (Kallmeyer *et al*., 2012). Cell numbers were found to decrease with depth and along a trophic gradient into the center of the gyre (D'Hondt *et al*., 2015). According to the low oxygen consumption rates, oxygen and nitrogen penetrate deep into the sediment columns and even down into the basaltic crust (D'Hondt *et al*., 2009; Fischer *et al*., 2009; D´Hondt *et al*., 2011; D'Hondt *et al*., 2015). As a consequence, microbial communities within the SPG sediments do not face electron acceptor-limitation (D'Hondt *et al*., 2015). However, the question arises, how they adapt on the energy limitation due to a lack of electron donors.

1.2 Viruses in marine ecosystems

Viruses are the most abundant biological entities on earth (Danovaro *et al*., 2008a) with estimated global numbers of 10^{31} (Breitbart & Rohwer, 2005). They were firstly discovered and described in the early years of the last century by Twort (1915) and d'Herelle (1917). Consisting of a nucleic acid that is surrounded by a protein capsule,

viruses are infectious agents (Weinbauer, 2004), exhibiting a replication that relies on the metabolism of its host. Viruses that infect prokaryotic cells are termed as bacteriophages or short phages (d'Herelle, 1917).

1.2.1 Viral life cycles

Until today, four different life styles of viruses have been identified: the lytic, lysogenic, pseudo-lysogenic and the chronic life cycle. Among them, lytic and lysogenic infections are most often studied (Figure 2).

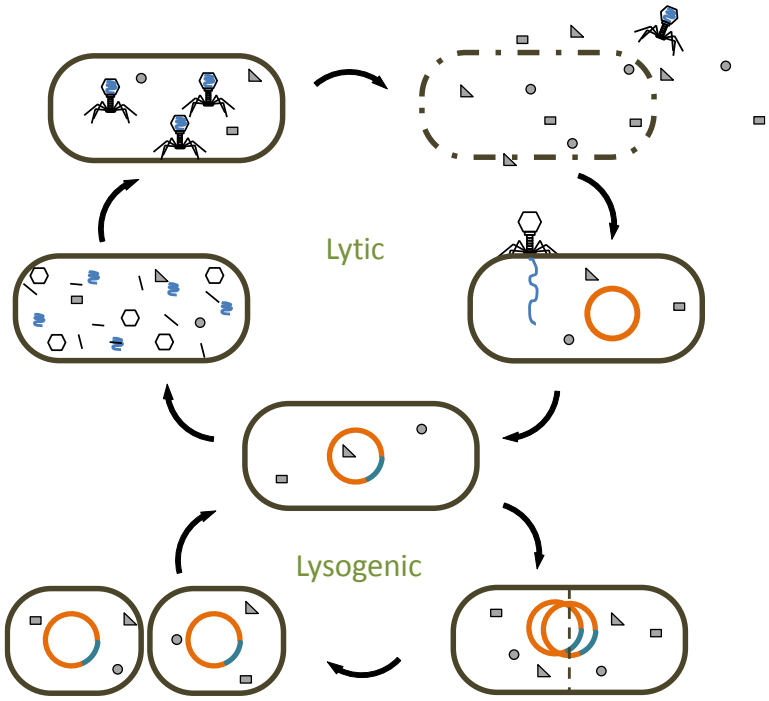

Figure 2. Simplified model of viral life cycles. After attachment of the phages and injection of its genome into the host cell, phages can follow either one of the two life cycles. Within the virulent reproduction, phages are rapidly produced by redirecting the host's metabolism. Subsequent self-assembly of the phage particles and production of lysing enzymes (Rohwer *et al.*, 2014) leads to cell lysis and the release of new phage particles as well as cell debris. During the lysogenic life cycle, the phage genome is integrated into the host genome and consequently reproduced by cell division. Re-entering the lytic life cycle can be induced by stress factors for the host.

In both cases, the first steps for an infection are attachment of the phage on the host's surface followed by an injection of the nucleic acid into the cell (Danovaro *et al.*, 2008a). During the lytic life cycle, phages immediately redirect their host's metabolism for production of new viruses, leading to a fast lysis of the cell. In contrast, if the

phages follow the lysogenic life cycle, their genome is integrated into the cell genome. Replication of these "prophages" takes place by the ongoing cell division (Weinbauer, 2004). Prophages remain in this status until unfavorable conditions for the host trigger an induction, which leads to virus production. Stress factors can e.g. be UV radiation, chemical or other physical factors (Danovaro *et al*., 2008a). Induction by the addition of the antibiotic mitomycin C is a common method for *in vivo* experiments (Jiang & Paul, 1996; Chen *et al*., 2006).

1.2.2 Different strategies in marine sediments dependent on environmental conditions

Entering either of the viral life cycles depends on several different factors, but is supposed to rely mostly on the environmental conditions. Reasons for this are direct connections between microbial and viral production (Glud & Middelboe, 2004) as well as successful virus-host encounters that can only occur with certain cell densities (Weinbauer, 2004). Lytic infection of the host in sediments has most often been reported for active sites and surface sediments that receive sufficient supply of substrates such as tidal flats and continental margin sites (Hewson & Fuhrman, 2003; Glud & Middelboe, 2004; Danovaro *et al*., 2008b; Siem-Jørgensen *et al*., 2008). Phages thereby might benefit from the current metabolic status of their hosts and use the ability to produce high amounts of new phage particles (Weinbauer, 2004). This theory is also known as "killing the winner" (Thingstad & Lignell, 1997). For example, it was shown that virus infections increased on the dominating prokaryotic strains in algae blooms (Hennes *et al*., 1995). Consequently, viruses have an influence on the prokaryotic diversity. Moreover, viruses contribute to the production of dissolved organic material by lysing the cells ("viral shunt"), which can further serve as nutrients for other microorganisms (Wilhelm & Suttle, 1999).

In contrast, lysogeny is supposed to be favorable in habitats with lower virus-host encounters and substrate availability (Fuhrman, 1999), e.g. subsurface sediments, where fresh organic material can only be transported via diffusive processes or in oligotrophic regions with low primary production within the water column (Jahnke, 1996; Billerbeck *et al*., 2006; *et al*., 2012). Under such disadvantageous conditions, ongoing production of new viruses might possibly result in extinction of the host

population or the prokaryotes might not be able to produce enough phage particles for further proliferation of the viruses. This is in accordance to previous studies on the water column (Weinbauer & Suttle, 1999). Here, the authors found lower burst sizes in open ocean waters with high UV radiation compared to coastal systems exhibiting higher turbidity.

1.2.3 Virus-to-cell ratios and special occurrence in SPG sediments

Several studies during the last few decades have reported on virus-to-cell ratios (VCR) as one factor for estimating the impact of viruses onto microbial communities (Danovaro *et al*., 2008b). In general, VCR in surface sediments lay between 0.2 and 98 (Hewson & Fuhrman, 2003; Mei & Danovaro, 2004). For subsurface sediments, increasing virus-to-cell ratios with depth were only reported in a few studies (Figure 1, (Bird *et al*., 2001; Mei & Danovaro, 2004). In a recent study, Engelhardt and colleagues (Engelhardt *et al*., 2014) compared several sites at continental margins and open oceans. They confirmed the initial findings, but could additionally determine an inverse correlation with the trophic status of the investigated habitats. Interestingly, for sediments of the SPG the VCR even reached a point, where the viral biomass might exceed that of the prokaryotic biomass. As viruses consist of easily degradable organic material, this fact arises interesting questions of a putative utilization of virus particles by indigenous prokaryotes, especially for an environment as inhospitable as the SPG.

1.3 Thesis outline

The aim of this study was to investigate the impact of viruses on deep-subsurface communities with special focus on the South Pacific Gyre. Sediment samples from deep sea drilling are always limited in volume. As shown by a previous study, tidal flat sediments can serve as model for the deep biosphere (Engelen & Cypionka, 2009). Based on this knowledge, the establishment of new methods can first be applied on easily accessible tidal-flat sediments such as Site Janssand. Thus, the precious samples of the International Ocean Discovery Program (IODP) can be kept until the main measurements.

Consequently, in the first study of this thesis, experiments were performed along a 4 meter sediment core of Site Janssand. Viral production rates were determined by the dilution method and by incorporation of radiolabeled ^{3}H-thymidine in order to

evaluate the impact of viruses on the microbial community in this specific habitat. Pros and cons of both methods are discussed in the manuscript and presented together with estimates of virus-induced prokaryotic mortality. Originally, it was planned to determine putative viral production in South Pacific Gyre sediments, too. However, as viral production measurements with highly active Janssand sediments already turned out to be rather challenging, further experiments with SPG sediments were not performed due to the low cell numbers.

Virus-prokaryote interactions in deep-sea sediments were analyzed in South Pacific Gyre samples due to previous findings of high virus-to-cell ratios in deeper sediment layers (Engelhardt *et al*., 2014). Based on these results, in the two other studies of this thesis, we hypothesized a putative utilization of virus particles by indigenous microbial communities as an adaptation to the low substrate availability. Therefore, two different strategies were systematically pursued. First, exoenzyme activities were determined on bulk-sediment samples and SPG isolates. Aminopeptidase and phosphatase activities were tested by using model-substrates. Measurements of aminopeptidase activity showed that the precondition for our hypothesis was fulfilled. In order to detect potential feeding on viruses by prokaryotes in more detail, further experiments were performed. Thus, in the third study, starving isolates as well as sediment from the SPG were incubated with concentrated phages from *Rhizobium radiobacter* strain P007 as sole carbon source. The results of the three studies lead to a better understanding of the viral impact on microbial communities in subsurface sediments of tidal-flats as well as on the adaptational mechanisms of microorganisms under extreme energy limitation.

References are listed at the end of this thesis

2 Publications

2.1 Viral production in an anoxic subsurface sediment of the German Wadden Sea

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Under revision in Aquatic microbial ecology

Abstract

Viruses impact benthic microbial communities by infection and lysis of prokaryotes. The effect of this virus-induced prokaryotic mortality can be estimated by measuring viral production rates. In this study, we determined viral production rates within anoxic subsurface sediments down to 375 cm in a German tidal flat. Viral production was measured by two independent methods: i) the dilution method, by which the increase in viral numbers over time is measured after dilution, and ii) the radiolabeling method, that is based on the incorporation of ^{3}H-thymidine via prokaryotes into virus particles. Viral numbers (1-39 x 10^{9} virus-like particles cm^{-3}) as well as viral production rates determined by the dilution method (2-82 x 10^{7} virus-like particles cm^{-3} h^{-1}) decreased with depth and were amongst the highest ever measured for marine sediments. The estimated virus-induced prokaryotic mortality (6-115% d^{-1} of prokaryotic standing stocks) as well as continuous viral production over days and weeks in incubations with ^{3}H-thymidine suggest a major impact of viruses on the microbial community in this subsurface sediment. Application of the radiolabeling method for viral production rate measurements revealed that the incorporation of ^{3}H-thymidine into virus particles was slow and incomplete for sediment incubations as well as enrichment cultures from sediments. The comparison of the two methods showed that the dilution method was far more sensitive in this tidal-flat sediment as viral production could be detected after a few hours of incubation compared to several days to weeks with the radiolabeling method.

Introduction

In marine surface sediments, viruses are more abundant than prokaryotic cells (Danovaro & Serresi 2000, Glud & Middelboe 2004, Engelhardt *et al*. 2014). Viral numbers are highest in the top layers (10^7 to 10^{10} viruses cm^{-3}) and decrease with depth in accordance with the decrease of cells (Bird *et al*. 2001, Danovaro *et al*. 2008b, Carreira *et al*. 2013, Engelhardt *et al*. 2014). The total number of viruses is a result of viral production and decay as well as import and export processes. Viral production rates in surface sediments (< 30 cm) range between 10^6 and 10^8 viruses cm^{-3} h^{-1} (Hewson & Fuhrman 2003, Middelboe *et al*. 2006) and are often correlated to microbial activity and abundance (Middelboe *et al*. 2003, Danovaro *et al*. 2008b, Siem-Jørgensen *et al*. 2008). Few studies have investigated viral production in deeper, anoxic layers down to 1 m. Rates at sediment depths of 10-100 cm were similar to rates in surface sediments (Mei & Danovaro 2004, Middelboe & Glud 2006).

The dilution method is commonly used to measure viral production rates. By diluting the samples, the background of free virus and the amount of virus-host encounters are reduced and thus, viral production is based on infections prior to the dilution (Wilhelm *et al*. 2002). Subsequently, an increase of viral numbers can be followed during incubation for a few hours. However, this method has been suggested to overestimate viral production due to stress induction of viruses or stimulation of microbial activity during dilution (Bratbak *et al*. 1994, Middelboe & Glud 2006). Quantifications of increasing viral numbers in undiluted, homogenized sediment incubations or even undisturbed whole sediment core incubations have been used as an alternative method that might not stimulate virus production (Middelboe & Glud 2006, Siem-Jørgensen *et al*. 2008). Another measure of viral production rates is based on the incorporation of radiolabeled ^{3}H-thymidine into DNA (Steward *et al*. 1992). The radiolabeling method has been used extensively to study heterotrophic microbial production in water samples (Fuhrman & Azam 1982) and has been adapted for viral production by Steward *et al*. (1992). Viral production can be determined by measuring the increase of ^{3}H-thymdine radiolabel that is incorporated into viral DNA during viral replication within the host cell. The method requires the uptake and incorporation of the radiolabel into the viral genome by the cell, which might change depending on the environmental conditions and the physiological state of the host. Therefore, the

conversion factor used to relate label incorporation to viral numbers is highly influenced by the host cell physiology via the uptake and incorporation frequency, the community composition and viral genome size (Noble & Steward 2001). The method has been repeatedly used to measure viral production rates in pelagic samples (Steward *et al*. 1992, Steward *et al*. 1996, Helton *et al*. 2005) and has also been used for samples from the sediment-water interface (Danovaro *et al*. 2008b). However, it has never been reported for viral production measurements in anoxic sediments.

Measuring viral production allows for estimates of virus-induced mortality of prokaryotes and for assessment of the impact of viruses on microbial communities (Proctor & Fuhrman 1990, Steward *et al*. 1996). Virus-induced mortality in sediments can account for 2 to 336% of the prokaryotic standing stock per day (Hewson & Fuhrman 2003, Glud & Middelboe 2004, Siem-Jørgensen *et al*. 2008) and 12 to 138% of prokaryotic net production (Mei & Danovaro 2004, Middelboe *et al*. 2006, Danovaro *et al*. 2008a). Thus, viruses have a considerable impact on the benthic microbial community, e.g., by shaping of the community structure and affecting biogeochemical cycling via the viral shunt (Proctor & Fuhrman 1990, Steward *et al*. 1996, Suttle 2007, Danovaro *et al*. 2008a). Although it is well documented that the effect of viruses on microbial communities in surface sediments can be substantial, studies on deep, anoxic sediments are scarce. In accordance to the decrease in microbial activity with depth (Parkes *et al*. 2014), viral production rates are expected to decrease as well.

In order to measure viral production in deep, anoxic sediments, a 4 m-core was taken from Site Janssand, located at the north-western coast of Germany. At this site, the organic carbon input from the water column into the sediments is high, and additionally, advective processes continuously supply fresh organic material into the upper layers (Rusch & Huettel 2000, Beck *et al*. 2011, Seidel *et al*. 2012). This leads to higher microbial activities in the surface compared to the diffusion-dominated sediments below with low oxygen penetration depths of 2-3 cm (Billerbeck *et al*. 2006, Seidel *et al*. 2012). In this study, we measured viral production rates along a sediment core from the surface to 375 cm by both the dilution and radiolabeling method. According to the high supply with organic material and high microbial activity in surface sediment, we expected viral production rates to be high in the surface and decrease with depth. As viral production rates in sediments measured with the

established methods of diluted, non-diluted or undisturbed incubations vary widely and sometimes have been assumed to result from biases by the methods itself, we anticipated to establish with the radiolabeling technique a fast, more sensitive and less intrusive method to measure viral production in marine sediments.

Material & Methods

Study site and sampling of sediments

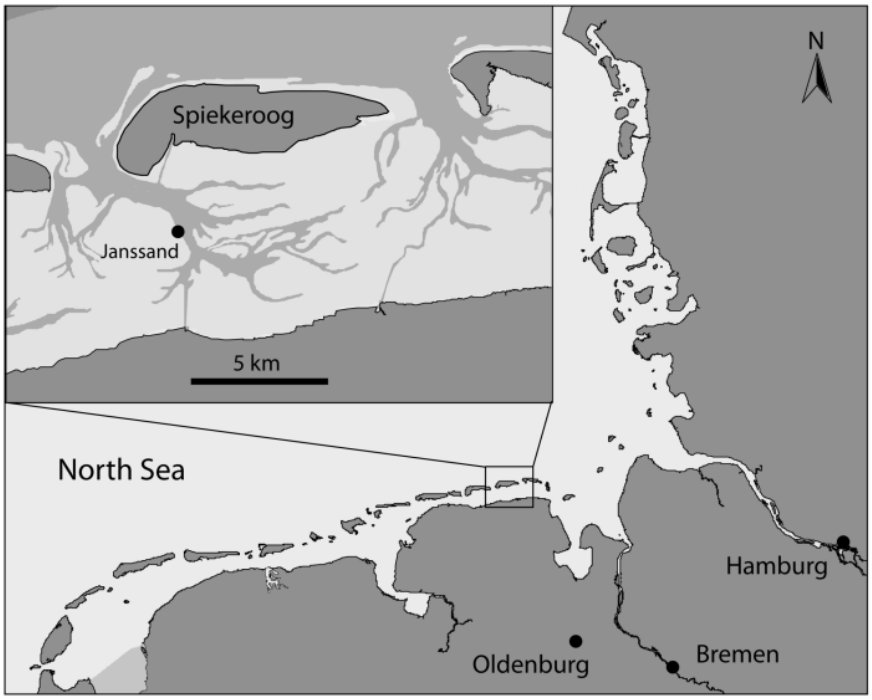

Figure 1. Location of the sampling Site Janssand in the back-barrier area of Spiekeroog island Northern German Wadden Sea. Figure taken from Seidel *et al*. (2012).

Samples were collected in July 2013 at the back-barrier tidal-flat area of Spiekeroog island, Site Janssand (Figure 1). Close to the water line at low tide, a 4-m long sediment core was retrieved by vibrocoring into an aluminum liner, which was immediately cut into half and sealed for transportation to the laboratory (Wilms *et al*. 2006). The temperature of the sediment in the top was 22 °C, at 5 cm depth 20 °C and 12 °C at 4 m depth measured just after retrieval of the core. Additionally, several short sediment cores were taken in plexiglass liners in close proximity to the long core to obtain undisturbed surface sediment and closed with stoppers on both ends. Transportation of the cores to the laboratory and subsampling was performed within a few hours after core recovery. Subsamples from the short cores were taken by pushing

the core up the liner and from the long core after cross-cutting the core at the required sections according to Table 1.

Table 1. Overview of subsamples taken from different cores of Site Janssand derived from short cores (S1-S4) or long core (L) for measurements of sulfate concentrations, sulfate reduction rates (SRR), methane concentrations, viral and prokaryotic counts and viral production rates.

Depth (cm)	Sulfate conc.	SRR & methane conc.	Viral & prokaryotic counts	Viral production rates
0.5	S1			
2		S2	S3	S4
3	S1			
5		S2	S3	S4
10	S1			
15	S1	S2	S3	S4
20	S1			
25	S1			
30	S1, L	L	L	L
50	L			
75		L	L	L
100	L			
125	L	L	L	L
150	L			
175	L		L	L
200	L			
225	L		L	L
250	L			
275	L		L	L
300	L			
350	L			
375			L	L

Samples for cell counts, viral counts, methane concentration and viral production measurements were sampled with cut-off syringes. Subsamples for viral counts were frozen immediately at -20 °C. Samples for cell counts were fixed with formaldehyde (2% final concentration) and stored at 4 °C for one day. For methane concentration measurements, sediment samples of 1 cm^3 were fixed with 2.5% sodium hydroxide in

glass tubes that were immediately closed with butyl stoppers. Syringes for viral production measurements were sealed with stoppers and immediately transferred to gas-tight bags that were continuously flushed with N_2. For sulfate reduction rate measurements, approximately 5 ml sediment from 2 to 125 cm (Table 1) was sampled in a glass tube which was closed with a plunger from a disposable syringe and a butyl stopper. Porewater samples for sulfate concentration measurements were gained from the sediments by either centrifugation or extraction by Rhizon samplers (Table 1), immediately gassed with CO_2 to strip sulfide and stored frozen at -20 °C.

Porewater constituents and sulfate reduction rate measurements

Methane concentrations were determined by gas chromatography and sulfate concentrations by ion chromatography. Sulfate reduction rates were measured in duplicate or triplicate incubations. Samples in glass tubes were injected with ^{35}S-sulfate and incubated for 7 hours at 15 °C, at which time the reaction was stopped by injection into 20% zinc acetate and freezing at -20 °C (Jørgensen 1978). Total reduced inorganic sulfur was analyzed by cold distillation (Kallmeyer *et al*. 2004), and sulfate reduction rates were calculated according to Jørgensen (1978).

Total cell counts

Formaldehyde-fixed sediment samples were washed twice with 1x phosphate buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 2.68 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 l water; pH 7.4) to remove the fixative. A mixture of PBS:ethanol (1:1 v/v, 0.5 ml) was added in the end and the samples were kept at -20° C until enumeration. Subsamples for cell counts were homogenized by manual shaking, diluted in PBS buffer and subsequently sonicated three times for 1 min. After 20 sec of sediment settling, 10 to 50 µl of samples were filtered onto a 0.2 µm pore size filter (Thermo Scientific Nalgene, Mexico). Filters were washed with PBS buffer, placed on a microscopic slide and stained with SYBR-Green I solution (Invitrogen). For enumeration, a minimum of 400 prokaryotic cells within 10 to 20 randomly chosen fields were counted with an epifluorescence microscope (Leica DMR, 400-fold magnification) and standard errors of the mean were calculated.

Viral counts

Viral counts were determined according to Danovaro *et al*. (2002) and Engelhardt *et al*. (2014) with a few modifications. In detail, 0.5 cm^{-3} of sediment was suspended in sodium pyrophosphate buffer (10 mM final concentration) and shaken for 15 min at room temperature. Samples were sonicated for 3 min, interrupted by manual shaking for 30 sec every minute and finally centrifuged at up to 3400 x g to settle sediments particles. The supernatant was filtered through a 0.2 µm syringe filter. Subsequently, sediment pellets were resuspended with 4 ml TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.4). After centrifugation, the second supernatant was 0.2 µm-filtered and combined with the first. Viral extracts were filtered onto 0.02 µm Anodisc filters (13 mm, Whatman, England). Filters were washed with TE-buffer and mounted onto a microscopic slide using 3-4 µl Moviol solution (Fluka, Switzerland). Virus-like particles were stained with SYBR-Green I and counted as described for cell counting with an Olympus BX51 microscope (600-fold magnification) standard errors of the mean were calculated.

Viral production rates determined by the dilution method

After sampling, samples (5 cm^{-3}) were immediately diluted 1:10 in artificial seawater media that was prepared oxic for the surface layer and anoxic for the rest of the sediment samples. Media were prepared according to Süß *et al*. (2004) with a few adjustments: vitamins and organic carbon sources were omitted, for the anoxic medium, sulfide was used as reducing agent and resazurine served as redox indicator. For the anoxic incubations, the glass vials were sealed under N_2/CO_2 (90:10, v/v) with butyl stoppers and incubated under anoxic conditions in the dark at 15° C. Additionally, one killed-control incubation was prepared with formaldehyde (final concentration of 2%). Subsamples of sediment slurries for viral counts (1 ml) were collected by syringes through the stoppers regularly during 24 h and stored at -20 °C until extraction and counting as described above. Production rates were calculated from slopes of linear regression lines of viral numbers and standard errors from linear regression lines of counts vs. time (r^2 = 0.8-1.0). A 6 hours interval was selected for all investigated depth layers. Applying an assumed burst size of 14, which had been determined with Baltic

Sea sediment previously (Glud & Middelboe 2004), and our viral production rates, virus-induced bacterial mortality was estimated, based on the cell counts.

Viral production rates by ^{3}H-thymidine incorporation

For comparison with the dilution method, rate measurements for viral production by ^{3}H-thymidine incorporation were also done in diluted incubations. After sampling, sediment (2 cm^{-3}) from the same depths as described above was added to 27 ml anoxic artificial seawater medium without vitamins (Widdel & Bak 1992). Three glass vials for each depth were inoculated with sediment and sealed under N_2/CO_2 (90:10, v/v) with butyl stoppers: one vial was amended with glucose as substrate (final concentration 5 mM) and two vials were incubated without any additive. Additionally, one killed-control incubation was prepared with formaldehyde (final concentration of 4%) and glucose. Approximately 740 kBq ^{3}H-thymidine (methyl-^{3}H, 2.22-3.33 TBq $mmol^{-1}$) was added to each vial. The vials were incubated at 15 °C and subsampled by syringes through the stoppers for a maximum of 10 days for all incubations with glucose and unamended incubations from 2 to 75 cm and for a maximum of 22 days for unamended incubations from 125-375 cm. Killed controls were treated equally to incubations from the deeper sediment layers. Subsamples of 1 ml were stored at -20 °C until further processing.

Virus extractions followed the protocol described above. For counting of radioactive decay, 7-8 ml of virus extract was filtered onto 0.02 µm Anodisc filters (13 mm, Whatman, England) and washed with 3 ml TE-buffer. Filters were transferred to scintillation vials and 1 ml 1 M HCl was added. Vials were placed in a water bath at 100 °C for 1 h. After cooling, 2 ml of scintillation liquid were added and samples were counted in a scintillation counter for 15 min. Counts in disintegrations per minute (dpm) of killed controls were subtracted from counts of samples. Production rates were calculated from slopes and standard errors from linear regression lines of counts against time (r^2 = 0.8-1.0). Only data which increased linearly with time were included into the regression, as incorporation of ^{3}H-thymidine in samples was delayed in the beginning and in the end leveled off for most of our incubations.

Determination of the conversion factor for the radiolabel method

In order to determine a conversion factor for the experiments, at first the sediment incubations were used. In contrast to radioactivity counts of the virus fraction, microscopic viral counts did not increase throughout the incubation time probably due to viral decay of unlabeled viruses towards the end of incubation. Thus, fast-growing enrichment cultures with high substrate concentrations under controlled conditions were used to determine ^{3}H-thymidine incorporation and viral numbers at several time points during the incubation. Enrichment cultures were established in oxic and anoxic artificial seawater media from Site Janssand in July 2013 with 10 mM glucose (final concentration). Anoxic media was prepared according to Widdel & Bak (1992). For the glucose-fermenting culture a medium with a reduced sulfate concentration of 0.1 g l^{-1} $MgSO_4$ x 7 H_2O to prevent growth of sulfate reducers was used. The oxic medium was prepared according to Süß *et al*. (2004). The enrichment cultures had been transferred several times before the start of the experiments so that no sediment particles were left. For the cultures of aerobically glucose-degrading prokaryotes, duplicate vials and one killed control with formaldehyde (4% final concentration) were incubated at 15 °C for 5 days on a shaker with 44 kBq ml^{-1} [methyl-^{3}H]-thymidine (Hartmann Analytic, 37 MBq ml^{-1}, specific activity: 2.22 - 3.33 TBq $mmol^{-1}$). The experiment with the aerobic enrichment cultures was repeated in an independent experiment in triplicates. For the cultures of anaerobic glucose-fermenting prokaryotes, triplicate vials and one killed control with formaldehyde were incubated at 15 °C for 8 days with 19 kBq ml^{-1} ^{3}H-thymidine.

Subsamples were withdrawn at several time points during the experiments, 0.2 µm filtered and stored at -20 °C. The incorporation of radiolabel into the virus fraction was determined as described above. Virus-like particles were counted via epifluorescence microscopy as described above. The conversion factor was calculated from the inverse of the slope of the regression line of moles ^{3}H-thymdine ml^{-1} vs. viruses ml^{-1} according to Steward *et al*. (1992).

Results

Sediment characteristics

Sediments of Site Janssand were dominated by sandy layers intercalated with thin mud-layers (Beck *et al*. 2009), with the first such occurrence at a depth of 55 cm in the analyzed core. Sulfate concentrations decreased with depth and sulfate was depleted at 125 cm (Figure 2). Sulfate reduction rates were highest at the top surface layer (332 nmol cm^{-3} d^{-1}) and steeply decreased with depth to 37 nmol cm^{-3} d^{-1} at 75 cm (Figure 2). Methane concentrations increased from the surface to 175 cm (106 to 460 nmol cm^{-3}) and remained within this range down to a depth of 375 cm (Figure 2).

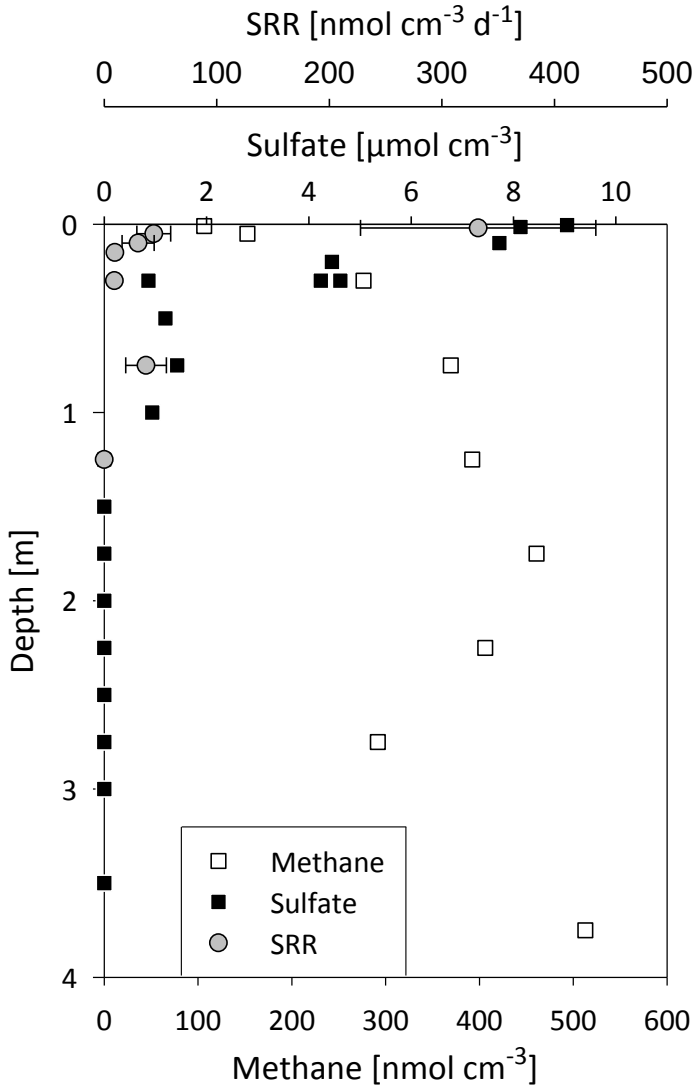

Figure. 2. Mean sulfate reduction rates (SRR) of duplicates and porewater sulfate and methane concentrations of Site Janssand.

Abundance of prokaryotes and viruses

Viral and prokaryotic cell numbers decreased steeply with depth in the upper 30 cm and to a lesser extent for the subsurface between 75 and 375 cm (Figure 3a). Viral counts ranged from 9 to 389 x 10^8 virus-like particles (VLP) cm^{-3} and cell counts from 3 to 36 x 10^8 cells cm^{-3} (Figure 3a, Table 2). Virus-to-prokaryote ratios were 9 to 59 in the uppermost 30 cm and 2 to 4 in the sediment layers below (Figure 3b).

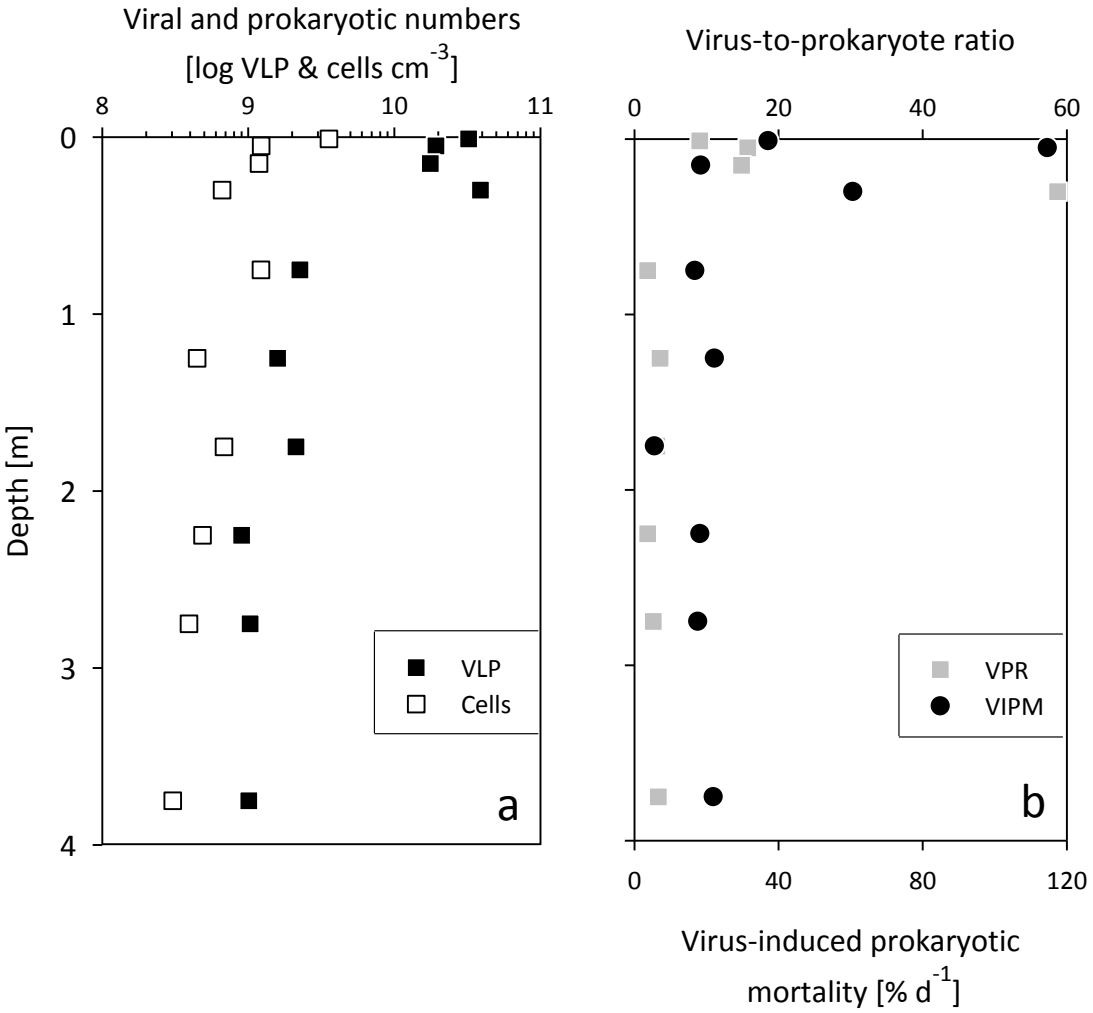

Figure 3. Numbers of viruses and prokaryotes (a), virus-induced prokaryotic mortality (VIPM) and virus-to-prokaryote ratios (VPR, b) of tidal-flat sediments of Site Janssand. Error bars are hidden behind the symbols for a.

Viral production rates determined by the dilution and radiolabeling methods

Pre-experiments with anoxic sediments for the ^{3}H-thymidine method showed that incubation times of a few hours did not result in label incorporation into viruses (data not shown). Instead incubations of several days were necessary to detect viral production (Figure 4). On the other hand for the dilution method, incubation times of several hours were used to detect the increase in viral numbers, while after the

increase viral production rates in general decrease or completely revert to viral decay (Dell'Anno *et al*. 2009). In order to determine viral production rates in this study, we used the dilution and radiolabeling method with diluted sediment incubations from 2 to 375 cm but with different incubation times of 6 hours and 4 to 22 days, respectively.

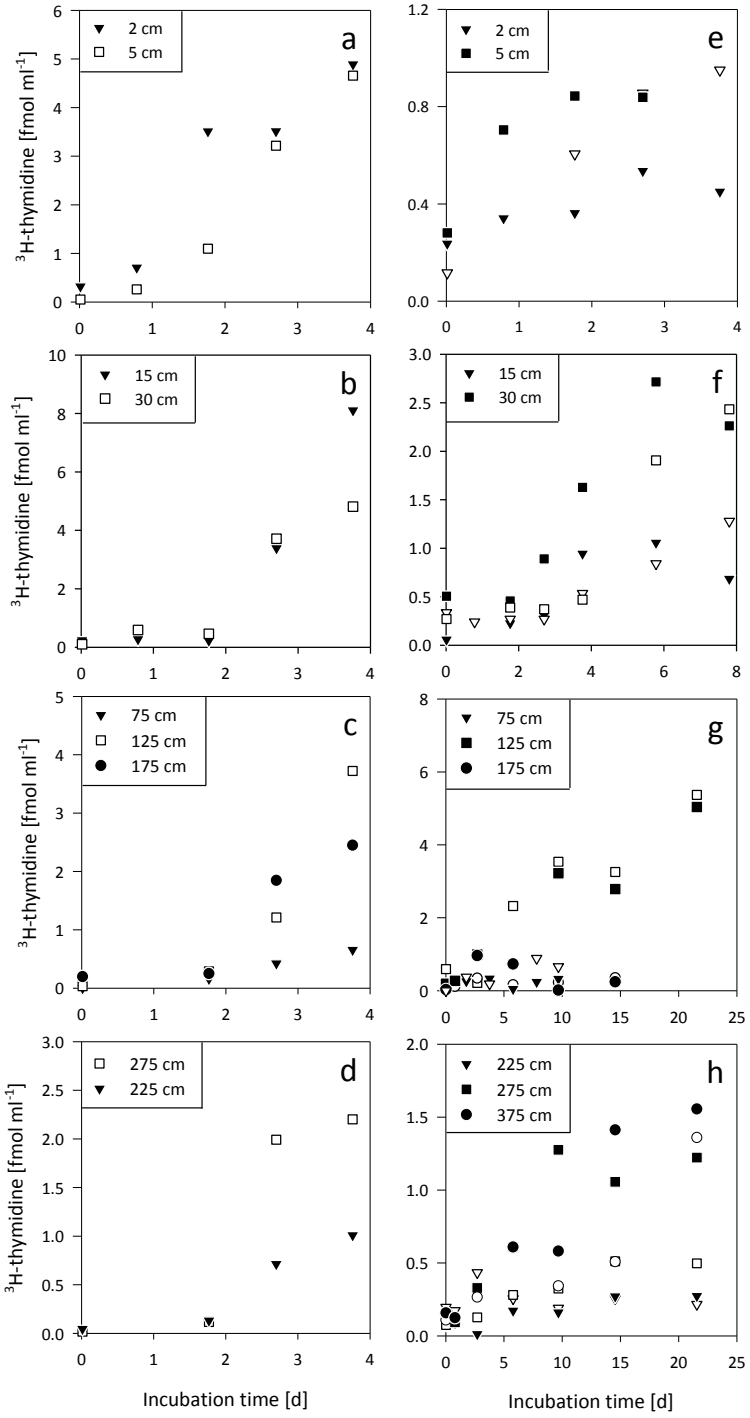

Figure 4. Label incorporation into viral fraction of sediment incubations from 2-375 cm with (a-d) and without glucose stimulation (e-f) determined by the radiolabeling method. The unamended incubations were performed in duplicates (open and closed symbols). Please note different scaling.

For the dilution method, viral numbers increased throughout the incubation periods for all sediment layers, while viral numbers in the killed control did not change over time, indicating that viral production was due to biological activity and was not due to the detachment of viruses from sediment particles. A linear increase in viral numbers was observed within the first 6 hours (Figure 5), while viral production rates eventually declined in some of the incubations (data not shown). Estimated viral production rates were highest in the surface layer with 8.2×10^8 VLP cm^{-3} h^{-1} and decreased exponentially with depth to 2.2×10^7 VLP cm^{-3} h^{-1} at 375 cm (Figure 6a).

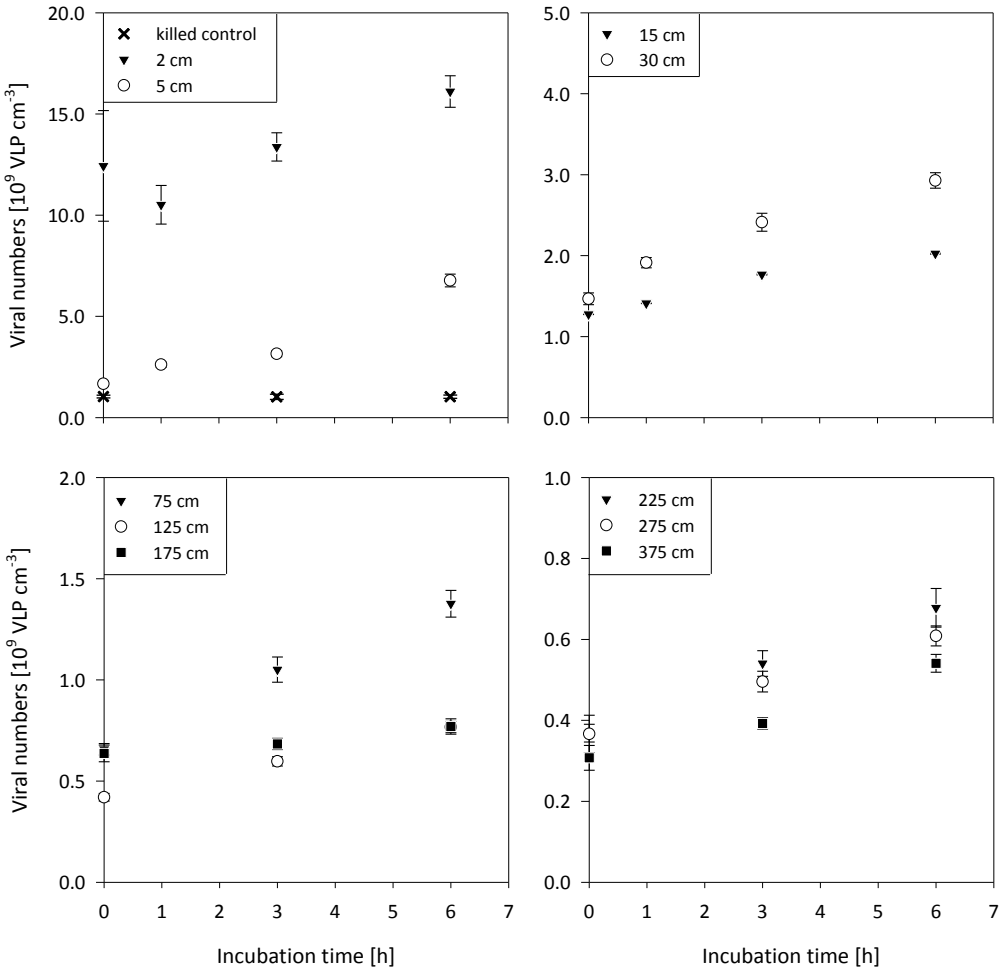

Figure 5. Viral counts for the first 6 hours of sediment incubations from 2-375 cm determined by the dilution method. Error bars represent standard errors of counts and if not shown, are hidden behind symbols. Please note different scaling.

In diluted sediment incubations with addition of ^{3}H-thymidine and glucose, ^{3}H-labeling of the virus fraction increased continuously within the first 4 days of incubation with rates of 2 to 30 fmol ^{3}H-thymidine cm^{-3} d^{-1} (Figures 4a-d & 6b). For the sediment incubations without glucose, the virus fraction was labeled more slowly (0.6 to 5 fmol

^{3}H-thymidine cm^{-3} d^{-1}; Figures 4e-h & 6b). For three depths (75, 175 and 225 cm) as well as the killed control incubations, no label incorporation was detected during incubation times of 10 to 22 days. For all other incubations, a steady initial increase in label incorporation was followed by a general decline that was probably due to decay of labeled viruses (data not shown). By glucose stimulation, viral production increased 4- to 17-fold. In these amended treatments, ^{3}H-thymidine incorporation into viruses was detected even for sediment samples where incorporation was below detection for the glucose-free incubations. However, ^{3}H-thymidine incorporation rates were still lowest in these depths, indicating that viral production rates were in general lower than at other depths.

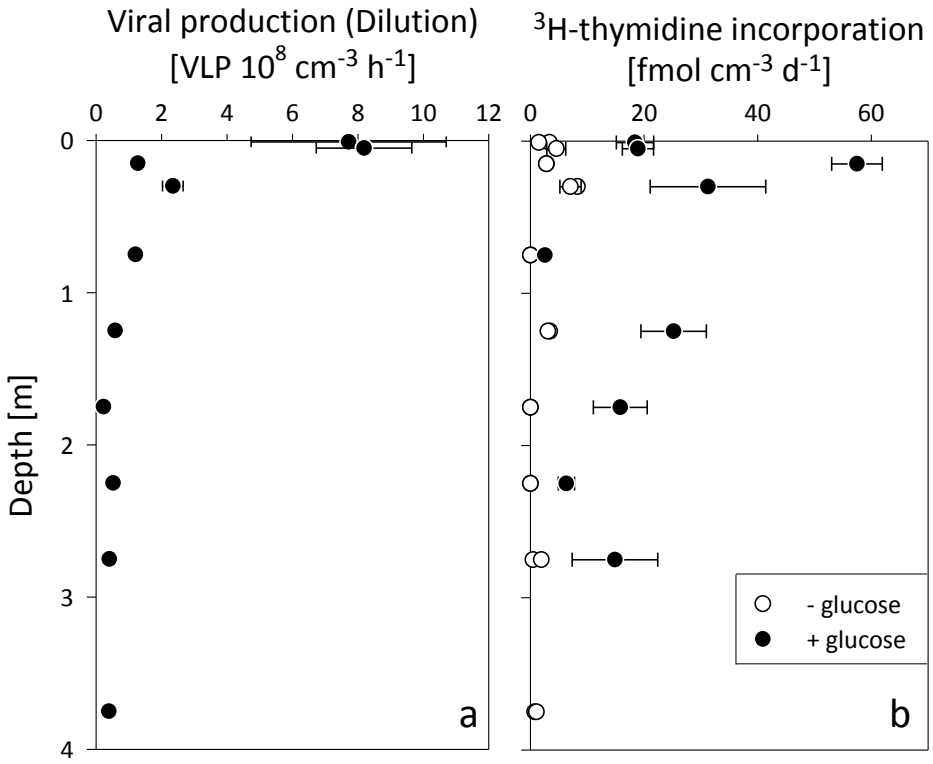

Figure 6. (a) Viral production rates of sediment incubations from 2 to 375 cm determined by the dilution method decreased exponentially. (b) ^{3}H-thymidine incorporation into viruses determined by the radiolabeling method with and without glucose amendment. Incubations of unamended sediments by the radiolabeling method were measured in duplicates. Thereby, ^{3}H-thymidine incorporation into viruses was not detected for sediment incubations of 75, 175 and 225 cm. Error bars represent standard errors of linear regression and if not shown, are hidden behind symbols.

Determination of conversion factors for the radiolabeling method

In order to convert ^{3}H-thymidine label incorporation quantities into numbers of viruses, a conversion factor is required. The determination of a new conversion factor

for this study was necessary because (i) this technique was applied for the first time to anoxic sediment samples, (ii) label incorporation into complete virus particles was measured in contrast to viral DNA only by Steward *et al*. (1992) and Danovaro *et al*. (2008b) and (iii) samples had to be incubated for several days in order to detect label incorporation in contrast to hours for water samples (Steward *et al*. 1992). Conversion factor determination requires experimentation that results in viral production greatly exceeding viral decay, i.e., the system cannot be in steady-state (Steward *et al*. 1992). We empirically determined conversion factors with two enrichment cultures from Janssand sediments, both supplemented with glucose (Figure 7). Estimated conversion factors in experiments with oxic and anoxic enrichment cultures differed by more than one order of magnitude (1×10^{22} to 7×10^{23} viruses mol^{-1}, respectively; Figure 7). A sulfate-reducing enrichment culture was also tested, but although viral counts and sulfide concentrations increased during the incubation, no incorporation of the label into viruses was detected (data not shown).

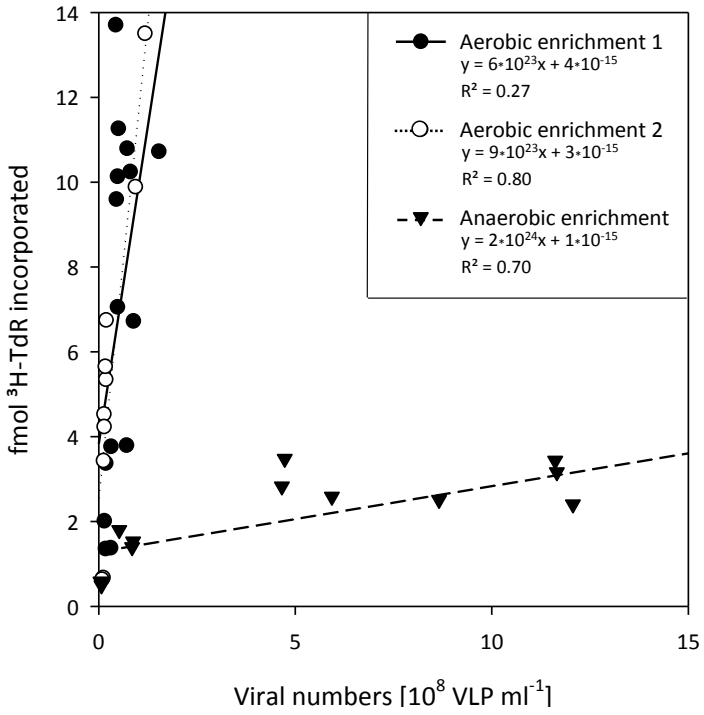

Figure 7. ^{3}H-Thymidine incorporation of aerobic and anaerobic enrichment cultures grown with glucose resulting in conversion factors of 1.7, 1.1 and 65 x 10^{22} viruses mol^{-1} for aerobic enrichment 1, aerobic enrichment 2 and anaerobic enrichment, respectively. Open and closed circles represent individual experiments.

Discussion

In this study, we have, to our knowledge for the first time, quantified viral production in anoxic sediments deeper than 1 m. Two methods for viral production rates were compared. For anoxic Janssand sediments, it was found that the dilution method was more sensitive than the radiolabeling method. High viral production rates in surface and subsurface sediments resulted in viral and prokaryotic turnover of a day to weeks.

Higher sensitivity of the dilution method compared to the radiolabeling method for viral production measurements

Our study showed that the dilution method was far more sensitive for determination of viral production rates in Janssand sediment than the radiolabeling method due to much shorter incubation times of 6 hours in contrast to a minimum of 4 days for the radiolabeling method (Figures 4 & 5). Short incubation times for the dilution method might be due to viruses released from cells that had already been infected prior to the start of the experiment (Wilhelm *et al*. 2002). In contrast, longer incubation for the labeling method might have been a result of the need for ^{3}H-thymidine uptake by the cells and subsequent incorporation into newly synthesized viral DNA.

The overall ^{3}H-thymidine incorporation into new viruses produced by sediment prokaryotes was very low for the enrichment cultures, as (i) ^{3}H-thymidine was not incorporated into viruses of sulfate reducers and (ii) the conversion factors of the glucose-degrading cultures were up to four orders of magnitude higher than empirical conversion factors described in the literature. Previously, conversion factors had been determined by Steward *et al*. (1992) with coastal seawater in unmanipulated samples (6×10^{20} viruses mol^{-1}) and diluted samples (2×10^{21} viruses mol^{-1}, determined during 6 hours of incubation) as well as by Danovaro *et al*. (2008b) using deep-sea samples (2×10^{20} viruses mol^{-1}). However, the high conversion factors of our study of 1 to 65×10^{22} viruses mol^{-1} show that approximately one molecule ^{3}H-thymidine was incorporated per virus particle. An average viral genome (50 kbp, 50% GC content) with 100% ^{3}H-thymidine label incorporation would result in a theoretical conversion factor of 2×10^{19} viruses mol^{-1}, as calculated by Noble & Steward (2001). The incomplete labeling of total viral DNA in our experiments might have resulted from intracellular isotope dilution by de novo synthesis or recycling of nucleotides (Noble &

Steward 2001) or from non-labeled viruses of prokaryotes that do not incorporate ^{3}H-thymidine into viral DNA (Gilmour *et al*. 1990, Winding 1992, Michel & Bloem 1993, Wellsbury *et al*. 1993 & 1994, this study). Conversion factors for radiolabeling methods to study both prokaryotic and viral production have been found to vary dependent on the environmental sample (Fuhrman & Azam 1982), manipulation of the samples (i.e., dilution; Steward *et al*. 1992, Teira *et al*. 2015) and prokaryotic generation times (Michel & Bloem 1993). Thus, they do not represent a constant which can be used independent of the environmental sample and experimental setting. Additionally, discrepancies in conversion factors may be introduced by differences (i) in labeling ratios of the thymidine pool, (ii) in individual label incorporation by pelagic vs. benthic microbial communities or (iii) in physiologies of prokaryotic groups (e.g., anaerobic vs. aerobic). Furthermore, differences in protocols, in label concentrations or incubation times might influence the conversion factor. Considering the above, it remains unclear if the conversion factor generated from the enrichment cultures can be used for accurate conversion of incorporation rates of the sediment incubations from Site Janssand. Thus, we did not convert our data, but present the ^{3}H-thymidine incorporation rates for comparison (Figure 6).

For Janssand sediment, ^{3}H-thymidine incorporation into viruses was detected for all investigated depths of Site Janssand (Figure 4). However, incubation times of several days were needed for all sediment layers, which might have altered the microbial activity and community composition. Some samples required additional stimulation with glucose in order to detect viral production, which resulted in incorporation rates that did not reflect in situ activity (Figure 4). However, it has been suggested that the dilution method overestimates viral production due to the stimulation of microbial activity by the dilution itself or stress induction of viral production by sample handling often indicated by a decrease in production rates and sometimes even decay following viral production (Bratbak *et al*. 1994, Middelboe & Glud 2006, Dell'Anno *et al*. 2009). Accordingly, viral production rates measured by the dilution method generally range among the highest rates, while those determined by the increase in viral numbers in undiluted sediment incubations are the lowest (Table 2, Middelboe & Glud 2006). Other factors that resulted in increased viral production rates are increase in temperature and organic carbon addition (Siem-Jørgensen *et al*. 2008). Thus, for our

sediment incubations at 15 °C, an overestimation of viral production by temperature increase during incubation was not likely for the surface sediment with in situ temperatures of 20-22 °C but might have occurred in deeper sediment layers as the temperature at the bottom of the core was 12 °C.

Table 2. Comparison of viral and cell numbers, virus-to-prokaryotes ratios (VPR) and viral production rates including the method used for analysis from unstimulated sediment incubations of different locations.

Location	Depth (cm)	Viral numbers (10^8 VLP cm^{-3}) (10^8 VLP g^{-1})*	Cell numbers (10^8 cells cm^{-3}) (10^8 cells g^{-1})*	VPR	Viral production rate (method) (10^7 VLP cm^{-3} h^{-1}) (10^7 VLP g^{-1} h^{-1})$^{\$}$	Ref.
Site Janssand, tidal flat, Germany	2-375	9.0-389	3.0-36	2-59	2.2-82 (dilution)	(1)
Southern California Bight, USA	0-1	2.0-25	0.023-0.25	10-98	28-50 (dilution)	(2)
Mediterranean Sea	0-1	2.1-25	9.3-29	0.2-0.9	1.3-16 (dilution)	(3)
Gulf of Manfredonia, Italy	0-1	6.7	14	0.5	1.5 (dilution)	(3)
	10-20	5.0	4.4	1.1	1.6	
	90-100	6.8	5.3	1.3	2.7	
Coastal to deep sea, world wide	0-1	1.0-35.9*	-	-	0.2-33$^{\$}$ (dilution)	(4)
Continental shelf to deep sea, Mediterranean Sea	0-1/ 15-16/ 25-30	5.6-18*	1.1-6.5*	1.1-9.4	1.9-61$^{\$}$ (dilution) 1.0-13.5$^{\$}$ (decay)	(5)
Freshwater lake, Austria	0-1	43-72	19-35	0.9-3.2	bd-22.2 (decay)	(6)
Coastal, Øresund, Denmark	0-10	1.5-3.8	0.13-0.22	11-17	0.18-0.67 (1-5 cm, undil. inc.)	(7)
Deep sea, Sagami Bay	0-15	1-23	0.29-1.3	5-35	0.17 (undil. inc.)	(8)
Continental margin, Chile	0.5-2	-	-	-	0.27-0.60 (undil. inc.)	(9)
	20-25	-	-	-	0.23	
	80-90	-	-	-	0.18	
Coastal, Øresund, Denmark	6-10	1.1-9.5	1.1-3.2	-	0.52-1.7 (undil. iInc.)	(10)
	0-0.5	2.8-12	1.7-3.9	-	0.35-1.1 (intact core)	
Deep sea, Porcupine Seabight	400-9600	0.049-1	0.0098-0.38	2-6	-	(11)
	1330 & 7980	-	-	-	Decreasing viral numbers (undil. inc.)	

References: (1) present study, (2) Hewson & Fuhrman 2003, (3) Mei & Danovaro 2004, (4) Danovaro *et al*. 2008b, (5) Corinaldesi *et al*. 2010, (6) Fischer *et al*. 2003, (7) Glud & Middelboe 2004, (8) Middelboe *et al*. 2006, (9) Middelboe & Glud 2006, (10) Siem-Jørgensen *et al*. 2008, (11) Middelboe *et al*. 2011

undil. inc.: undiluted incubations, bd: below detection, *viral and cell numbers in g^{-1} dry sediment, $^{\$}$virus production rates in viruses g^{-1} dry sediment h^{-1}

In conclusion, our study demonstrates that for future studies it is extremely important to evaluate different methods and handling of sediment in order to find the most accurate protocol to determine viral production rates in the selected sediment type.

High viral production impacts the microbial community

High viral and cell numbers in Janssand sediment were similar to the numbers reported in surface sediments from the tidal flats of the Scottish Dunstaffnage Bay, Mediterranean Sea shelves and several deep-sea sites (Table 2, Mei & Danovaro 2004, Corinaldesi *et al*. 2010, Carreira *et al*. 2013, Engelhardt *et al*. 2014). Similarly, viral production rates of up to 8 x 10^8 VLP cm^{-3} h^{-1} in the uppermost sediment layers of Site Janssand were among the highest ever measured (Table 2). As viral production rates in coastal surface sediments have been found to correlate to microbial activity and abundance (Middelboe *et al*. 2003, Danovaro *et al*. 2008b, Siem-Jørgensen *et al*. 2008), high viral production rates were in accordance to the high cell numbers and activities in Janssand sediments (Figures 2 & 3, Gittel *et al*. 2008, Beck *et al*. 2009, Beck *et al*. 2011). The microbial activity in surface sediments of Northern German tidal flats is stimulated by high input of organic matter from the water column, advective processes, bioturbation and the drainage of porewater through the upper sediment of the intertidal creek bank during low tide (Poremba *et al*. 1999, Beck *et al*. 2009, Riedel *et al*. 2010, Seidel *et al*. 2012). Decreasing viral production rates in relation to depth along the sediment redox gradient likely reflected the decrease in microbial activity, which in general is controlled by declining availability of organic matter with depth (Figures 2 & 6, Seidel *et al*. 2012).

High viral production rates have been found with the dilution method for surface sediments of the shelves of the Southern California Bight, the Mediterranean Sea and deep-sea sediments of the Black Sea (Table 2, Hewson & Fuhrman 2003, Danovaro *et al*. 2008b, Corinaldesi *et al*. 2010). For deeper, anoxic layers of the Gulf of Manfredonia, viral production rates were one order of magnitude lower at 15 and 95 cm depth compared to the surface which is similar to the 40-fold decrease over 375 cm depth at Site Janssand (Figure 6, Table 2, Mei & Danovaro 2004). However, it remains unclear whether viral production can be sustained in sediments with low microbial activity. In undiluted incubations of sediments from 13 and 80 meter below

seafloor of the eastern margin of the Porcupine Seabight, viral production could not be detected but instead viral numbers decreased with incubation time (Middelboe *et al*. 2011). The authors suggested that homogenization of the sediments exposed the viruses to degradation processes.

Estimates of viral turnover in Janssand sediments were between 0.7 and 7 days. Similar viral turnover times with 5 to 6 days were reported for deep-sea surface sediment of Sagami Bay, Japan, and estuarine surface sediment of Øresund, Denmark, with 1 to 28 days (Glud & Middelboe 2004, Middelboe *et al*. 2006, Siem-Jørgensen *et al*. 2008). In contrast, sediments in the Southern California Bight showed extremely short viral turnover times of 0.04 to 0.2 days which resulted in high prokaryotic mortality by viruses of 96-336% d^{-1} of the prokaryotic standing stock (Hewson & Fuhrman 2003). Virus-induced prokaryotic mortality in Janssand sediment, estimated from viral production rates and an assumed burst size of 14 (Glud & Middelboe 2004), was between 6 and 115% d^{-1} of the prokaryotic standing stock (Figure 2b). They are similar or up to twice as high compared to reports for coastal and deep-sea sediments where prokaryotic mortality was 2 to 60% d^{-1} (Glud & Middelboe 2004, Mei & Danovaro 2004, Middelboe *et al*. 2006, Siem-Jørgensen *et al*. 2008). The decrease in turnover of the microbial community below 30 cm to a relatively constant impact of viruses on prokaryotic mortality of 6-22% d^{-1} is in line with lower microbial activity and likely slower growth. As grazers are expected to be absent in anoxic sediments (Fenchel & Riedl 1970, Lee 1992), it can be assumed that viruses are the major mortality factor for prokaryotes in contrast to the water column where the contribution is shared on changing proportions between viruses and grazers (Fuhrman & Noble 1995, Weinbauer *et al*. 2003).

In summary, our results showed that the turnover of viruses and prokaryotes is similar in Janssand sediment ranging from days to weeks. Viral production rates, virus-to-prokaryote ratios and virus-induced prokaryotic mortality were higher in surface-near sediments suggesting that viral communities in the upper layers are influenced by the dynamic changes in the surface sediment (e.g., tides, waves, bioturbation, organic matter input). Viral production as deep as 3.75 m and continuous viral production in incubations with radiolabel over several days to weeks showed that viruses also have a major impact on deeply buried microbial communities.

Author contribution (This part was not included in the submitted manuscript)

FP: Concept, sampling, measurements, data analyses (calculations, creation of figures and tables), data interpretation, first draft

BE: Idea and concept, data interpretation and revision of the manuscript

SK: Measurements, data analysis (calculations), revision of manuscript

TE: Data interpretation and revision of the manuscript

HC: Data interpretation and revision of the manuscript

VV: Concept, sampling, measurements, data analyses (calculations), data interpretation and revision of the manuscript

Acknowledgements

This study was supported by grants from the German Science Foundation (DFG priority program "International Ocean Discovery Program") and was partly funded by the Danish National Research Foundation and the European Research Council (ERC), Advanced Grant MICROENERGY (grant agreement no. 294200), awarded to Bo Barker Jørgensen under the EU 7th FP. Thanks to Anja Grubert and Nicole Beier for help with sample processing. The authors thank Marion Pohlner and Saranya Kanukollu for research assistance in sampling and SRR measurements as well as Candice Raeburn for giving valuable comments. We also thank Uwe Winkler and Inga Kayser for technical support in the isotope laboratory. We are thankful to the two anonymous reviewers who helped to improve the manuscript.

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2.2 Microbial communities in deep-subsurface sediments of the South Pacific Gyre exhibit exoenzyme activities

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Submitted to Frontiers in Microbiology

Abstract

Microbial communities in deep-subsurface sediments face severe energy- and electron-donor limitation. The acquisition of nutrients is triggered by the excretion of enzymes that hydrolyze polymeric substances. As the production of exoenzymes is energetically cost-intensive, it would be interesting to know whether microbial communities in extremely oligotrophic subsurface sediments still exhibit measurable activities. In this study, we have exemplarily investigated phosphatase and aminopeptidase activities in subsurface sediments and on indigenous bacteria of the South Pacific Gyre (SPG) that were obtained from Expedition 329 of the International Ocean Discovery Program (IODP). We hypothesized that the activities are generally low, that proteins might be a potential food source for subsurface microorganisms and that the phosphatase activities are correlated to the phosphate concentrations. The tested sediment samples from various depths and SPG sites revealed aminopeptidase activities of 0.0002-0.4 nmol cm^{-3} h^{-1} and phosphatase activities of 0.04-3.6 nmol cm^{-3} h^{-1} which is in the range of previously investigated subsurface sediments. The calculated cell-specific phosphatase and aminopeptidase activities (0.15-866 and 0.009-28 fmol $cell^{-1}$ h^{-1}, respectively) were comparatively high and are probably biased by low cell numbers. Decreasing aminopeptidase activities with sediment depth indicated lower, but still detectable microbial activities within these layers. Thus, proteins might be degraded at lower rates and consequently preserved in deep-subsurface sediments. Total phosphatase activities increased with depth and were inversely correlated with phosphate concentrations within the tested sediment horizons. This activity is surprising as the investigated microbial communities are not exposed to phosphate-limitation even in sediment layers with lowest phosphate concentrations. Part of the phosphatase activity might be due to absorption of enzymes on sediment particles although retaining the enzyme activity over geological timescales is rather unlikely. Thus, our findings and the fact that all isolates exhibited both types of exoenzyme activities indicate an active production rather than preservation.

Introduction

Deep-subsurface sediments were originally proposed to contain 35-47% of the global biomass harboring approximately one third of all prokaryotic cells on Earth (Parkes *et al*., 1994; Whitman *et al*., 1998). These first calculations were overestimated as most studies on the deep-subseafloor biosphere have been performed at ocean margin sites (D´Hondt *et al*., 2013), characterized by high primary production in the water column (Jahnke, 1996) and relatively high microbial activities within the underlying sediments (D'Hondt *et al*., 2004). After including more oligotrophic oceanic provinces into the global estimates, the initial numbers were downsized to ~0.6% of Earth´s total biomass (Kallmeyer *et al*., 2012). However, the global cell numbers of deep-subseafloor sediments (2.9×10^{29}) are still as high as in all ocean waters (1.2×10^{29}), or soils (2.6×10^{29}, Kallmeyer *et al*., 2012). As for adjusting the global cell numbers, extremely oligotrophic marine environments obviously need to be included to study life under extreme energy limitation.

The most oligotrophic marine environments, the mid-ocean gyres, comprise ~48 % of the world´s oceans (D'Hondt *et al*., 2009) and are characterized by low primary production in the photic zone (Jahnke, 1996). Accordingly, only small amounts of organic matter reach the seafloor, resulting in sedimentation rates of 0.008-1.1 mm kyr^{-1} for the South Pacific Gyre (SPG, D'Hondt *et al*., 2009). This part of the “ocean deserts” was target of Expedition 329 that was conducted in the frame of the International Ocean Discovery Program (IODP) in 2010 (D´Hondt *et al*., 2010). First results of the expedition and the preceding site-survey revealed that oxygen and nitrogen penetrate deep into the sediments of the SPG and even down into the basaltic crust (D'Hondt *et al*., 2009; Fischer *et al*., 2009; D´Hondt *et al*., 2011; D'Hondt *et al*., 2015). The low organic carbon content at the seafloor diminishes oxygen consumption by aerobic respiration, which in turn leads to oxygenated sediments (Fischer *et al*., 2009; Røy *et al*., 2012). Due to the low substrate availability, cell numbers are orders of magnitudes lower than in sediments with equivalent water depths (Kallmeyer *et al*., 2012). Numbers of prokaryotes decrease with sediment depth and along the trophic gradient from the edge into the center of the gyre (D'Hondt *et al*., 2015). Røy and colleagues (2012) reported similarly low cell numbers and high oxygen penetration depths for North Pacific Gyre sediments. Thus, microbial

communities within sediments of mid-ocean gyres are not electron-acceptor limited, but rather restricted by electron-donor availability (D'Hondt *et al*., 2015).

Precondition for the prokaryotic uptake of substrates is the hydrolysis of polymeric substances, which is the rate-limiting step of organic matter remineralization (Boetius and Lochte, 1994; Lehman and O'Connell, 2002). Several bacteria release hydrolytic enzymes such as glucosidases or proteases into the environment. Before cellular incorporation, these enzymes degrade polymers such as chitin, cellulose or proteins into their building blocks like sugars or amino acids (Weiss *et al*., 1991). Thus, determining exoenzyme activities is used as a measure for carbon remineralization in marine systems (Arnosti, 2011). A common method to determine exoenzyme activities is based on the conversion of substrate analogues coupled to 4-methylumbelliferone (MUF) or 7-amino-4-methylcoumarin (MCA), respectively (Hoppe, 1983; Hoppe, 1993). For subsurface sediments, these kinds of measurements are rare. Using the same approach, Coolen & Overmann (2000) analyzed ancient Mediterranean sapropels and organic-lean intermediate clay layers. Their findings indicate that bacterial communities within the sapropels are metabolically active and can partly degrade the kerogenic sediment matrix.

In this study, we tested whether benthic microbial communities of the South Pacific Gyre exhibit exoenzyme activities. As the release of these exoenzymes is energetically cost-intensive for the cells, we expected to find rather low activities within the SPG sediments characterized by extremely low cell numbers and substrate availability. To test this hypothesis, we have investigated bulk sediment samples and bacterial isolates from the SPG recovered during IODP Exp. 329. Sediment samples derived from seven sites and three different depths, each along two transects into and out of the Gyre (IODP Sites U1365-U1371), the isolates were obtained from IODP Site U1371, at the edge of the SPG.

We have chosen aminopeptidase and alkaline phosphatase (short: phosphatase) as model enzymes to be tested. Aminopeptidase activities are an indicator for carbon utilization via protein degradation (Boetius & Lochte, 1994; Boetius, 1995; Boetius *et al*., 1996). We hypothesized that proteins might be a bioavailable carbon source in these sediments. In our own investigations on the role of viruses within SPG sediments, we found dramatically increasing virus-to-cell ratios with depth, leading to

the conclusion that a large amount of carbon was virus-bound rather than cell-bound (Engelhardt *et al*., 2014). In former studies, it was reported that bacteria can enzymatically degrade virus particles (Fujioka *et al*., 1980; Mojica & Brussaard, 2014) that are composed of nucleic acids wrapped in protein capsids (Weinbauer, 2004). Cliver & Herrmann (1972) proofed e.g. the utilization of viral protein capsules of enteroviruses as a potential food-source after degradation by aminopeptidases.

Phosphatase activities were exemplarily investigated to test whether the microbial communities can respond to decreasing phosphate concentrations with sediment depth as determined for all SPG sampling sites (D'Hondt *et al*., 2015). Engelen *et al*. (2008) e.g. found increasing phosphatase activities along a 265 meter long sediment column of the Juan de Fuca Ridge and interpreted this as a compensation for phosphate deficiencies in phosphate-poor layers. Due to the low activities expected, all measurements were performed under elevated temperature higher substrate concentrations and diminished pressure as compared to the *in-situ* conditions. Thus, all exoenzyme activities are referred to be potential.

Material and Methods

Study site and sample collection

Sediments were collected during an expedition of the International Ocean Discovery Program (IODP Exp. 329) with the research vessel JOIDES Resolution. Seven sites along two transects were sampled there and back again: (i) from the western edge of the South Pacific Gyre into the center (U1365 to U1368) and (ii) from the center southwards towards an upwelling region (U1371) that served as a reference site (Figure 1; D´Hondt *et al*., 2011; D'Hondt *et al*., 2015). Sediment cores were drilled via piston coring and directly processed after retrieval. Potential contamination with drilling fluids was tested by using perfluorocarbon tracers (D´Hondt *et al*., 2010) excluding contamination in the middle of the cores (Lever *et al*., 2006). Thus, about 70 cm^3 of sediment were subsampled from the most pristine centers of whole-round cores. Samples were taken at all sites from different depths and stored at 4 °C under nitrogen atmosphere until further processing.

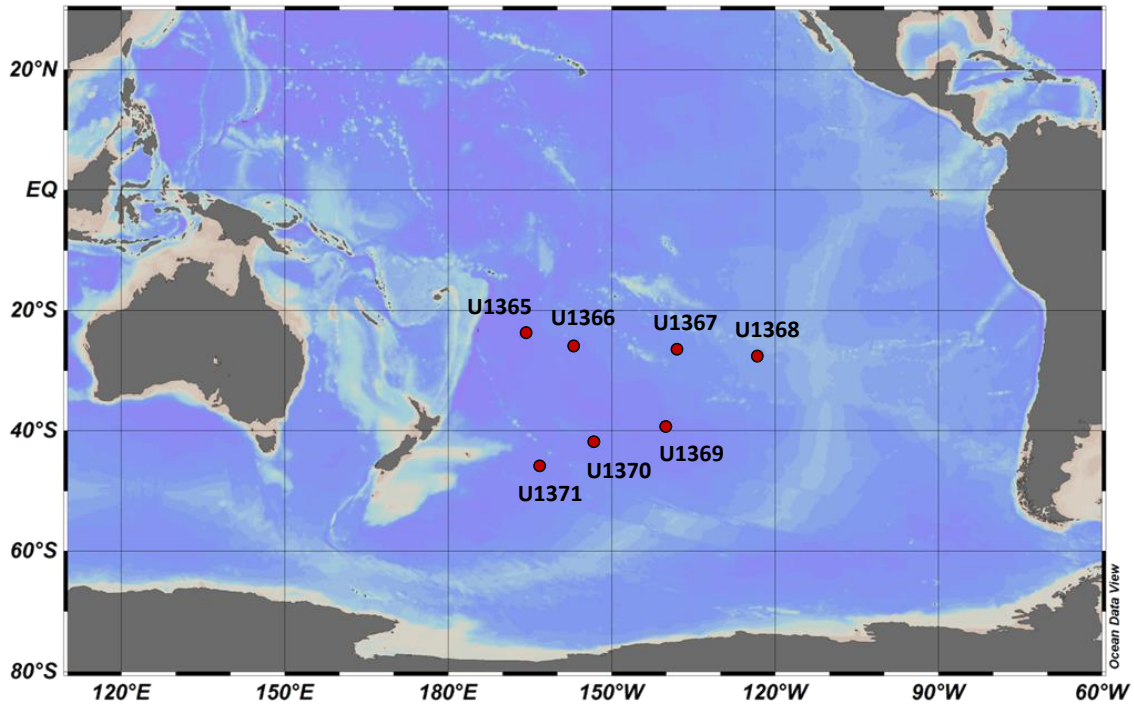

Figure 1: Map of the South Pacific Ocean. Sites U1365 to U1371 of IODP Exp. 329 to the South Pacific Gyre are indicated. The map was created with Ocean Data View (Schlitzer, R., Ocean Data View, http://odv.awi-bremerhaven.de, 2004).

Enrichment cultures and bacterial isolation procedure

Enrichment cultures for the isolation of pure cultures were setup onboard the RV JOIDES Resolution in 1:10 dilution series using three different media to target both, aerobic and anaerobic heterotrophs as well as autotrophs. Artificial seawater medium was prepared according to Süß *et al*. (2004) for oxic incubations and Batzke *et al*. (2007) for anoxic enrichments, both containing 36 different monomeric carbon sources (0.1 mM final concentration). The medium for autotrophic enrichments was prepared as the anoxic medium, except the addition of the substrate mixture. The headspace was flushed with hydrogen (90%) and carbon dioxide (10%). The cultures were incubated at 4 °C for approximately one year and transferred afterwards to 15 °C to increase growth rates. All enrichment cultures were screened for growth by means of epifluorescence microscopy using SYBR-Green I as a fluorescent dye (Lunau *et al*., 2005). After screening, subcultures were setup for all positive samples. The oxic enrichments were transferred onto agar plates, while the anoxic and autotrophic enrichments were subcultured in deep agar dilution series applying the respective media. Colonies were transferred and four times subcultivated onto fresh agar plates or into deep agar dilution series for further purification. Partial 16S rRNA sequences

(>1300 bp) of the isolates used in this study were compared to those in GenBank using the BLAST function (Altschul *et al*., 1997). All sequences have been deposited in the EMBL database under accession numbers LN898660 – LN898663.

Exoenzyme assays

All measurements and calculations of exoenzyme activities were performed according to the protocol of Coolen & Overmann (2000) with a few adjustments. Phosphatase and protease activities of pure cultures and microbial communities in SPG sediments were determined by using the fluorescent labeled MUF-phosphate (MUF-P) and MCA-leucine (MCA-Leu) as substrates analogues. For each of the seven sites, surface sediments, intermediate horizons and deepest layers were chosen. For Site U1366 no surface sediment was available and for Site U1369, only surface and bottom sediments were tested. As the sediments of Site U1371 were anoxic after the upper meter, measurements of intermediate and deepest layers of this site were performed within an anaerobic chamber. For measurements under oxygen-free conditions, anoxic artificial seawater was prepared and the reaction vials were sealed with butyl rubber stoppers.

The fluorophores were dissolved in 1:100 ethylenglycol (v/v). Sediments (diluted 1:10 with artificial seawater) or cultures were transferred into sterile vials containing stirring bars (final volume 1.4 ml). The enzymatic reaction was started by adding 100 µl of the respective substrate analogue (final concentration 50 or 550 µM for MCA-Leu or MUF-P, respectively). All phosphatase activities were measured under saturation conditions for the substrate analogue MUF-P (Figure S1a). Aminopeptidase activities were determined under conditions below the saturation level, but still not under substrate limitation (Figure S1b).

Samples were incubated at room temperature in the dark between 15 minutes and 24 hours. For sediments, incubation times had to be adjusted individually. Assays containing MUF-P were stopped with 100 µl NaOH (final concentration 35 mM) and 100 µl Na_4EDTA (final concentration 95 mM). Enzyme-activity measurements with MCA-Leu were stopped by the addition of 200 µl acetone. The samples were then transferred into 2 ml tubes and centrifuged for 5 min at 14000 rpm. 200 µl of supernatant was transferred into a microtiter plate and free fluorophores were

measured in a plate reader (excitation 380 nm, emission 460 nm, BMG Labtechnologies, Offenburg, Germany). All samples were measured in triplicates.

One blank, three controls as well as an internal and an external standard were set up for each assay. The blank contained artificial seawater (1.4 ml), ethyleneglycol (100 µl, diluted 1:100) and the respective stop-solutions (200 µl). Control 1 (autofluorescence of untreated sample) was composed of 1.4 ml sample, ethyleneglycol (1 µl) and 100 µl artificial seawater. Control 2 (autofluorescence of heat-inactivated sample) was prepared as control 1 except additional boiling prior to incubation for 20 minutes at 95 °C. Control 3 (background activity) was composed as described for the samples, but boiled prior to incubation for 20 minutes at 95 °C. All controls and standards were incubated, treated and measured as described above. External and internal standards for phosphatase activity (0.25-5 µM) and protease activity (2-50 µM) were compared and used to calibrate the measured values of the samples. The measurements were corrected for the blank and the controls. Raw data of phosphatase activities from IODP Site U1301 (Engelen *et al*., 2008) were newly processed to eliminate a calculation error (Figure S2). Cell-specific exoenzyme activities were calculated for the cultures by using direct counts or published cell counts for the respective sediment layers (D'Hondt *et al*., 2015).

Results

Increasing phosphatase activities correlated to declining phosphate concentrations in SPG sediments. Phosphatase activities could be determined for all sediment samples, except for those from the intermediate layer of Site U1366 (Figure 2a, b). The background phosphatase activity was determined by heat-inactivation of the sediments. It ranged between 0.04 and 75.6 nmol cm^3 h^{-1} (Figure 2a), superimposing the phosphatase activity of the untreated sediments in most of the intermediate and bottom layers. Therefore, the background-corrected phosphatase activity could only be determined for the surface samples and some deeper layers (Figure 2b).

Overall, the corrected phosphatase activities differed by two to three orders of magnitude and ranged between 0.04-3.6 nmol cm^{-3} h^{-1}. Both, background and corrected activities showed no clear trend along the two transects from the edges to the center of the gyre, but an increase with sediment depth for most of the samples.

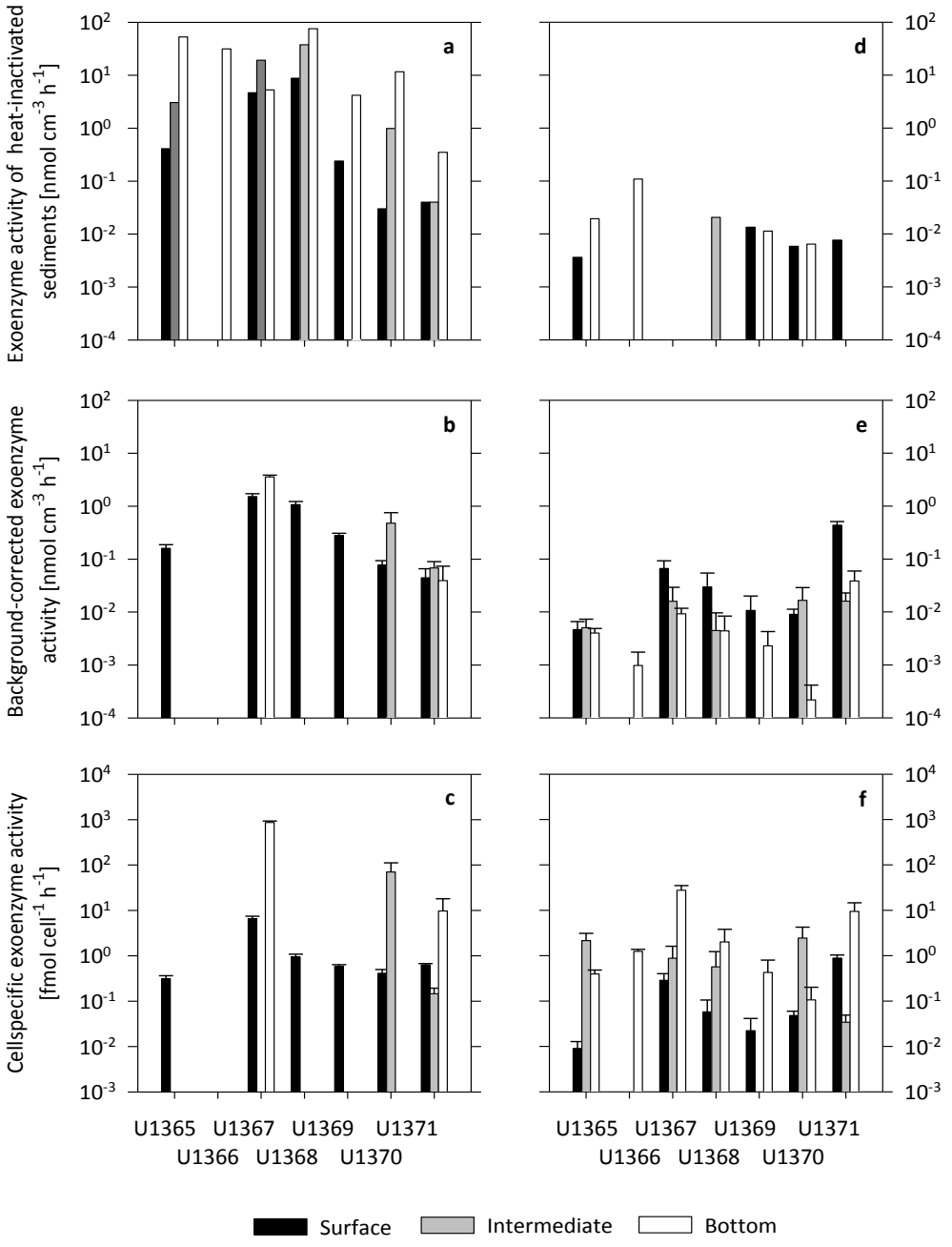

Figure 2: Exoenzyme activities of phosphatase (a-c) and aminopeptidase (d-f) from seven different sites and sediment horizons of the South Pacific Gyre sampled during IODP Exp. 329. Exoenzyme activities of heat-inactivated sediments (a, d) were determined to identify background activities. Background-corrected activities are displayed in b and e. Cell-specific activities (c, f) were calculated from the background-corrected activities and published cell numbers (D'Hondt *et al*., 2015).

At all sites, phosphatase activities were high in sediment layers with low phosphate concentrations and vice versa (Figure 3). Total phosphatase activities correlated inversely ($R^2 = 0.73$) to phosphate concentrations (Figure 4).

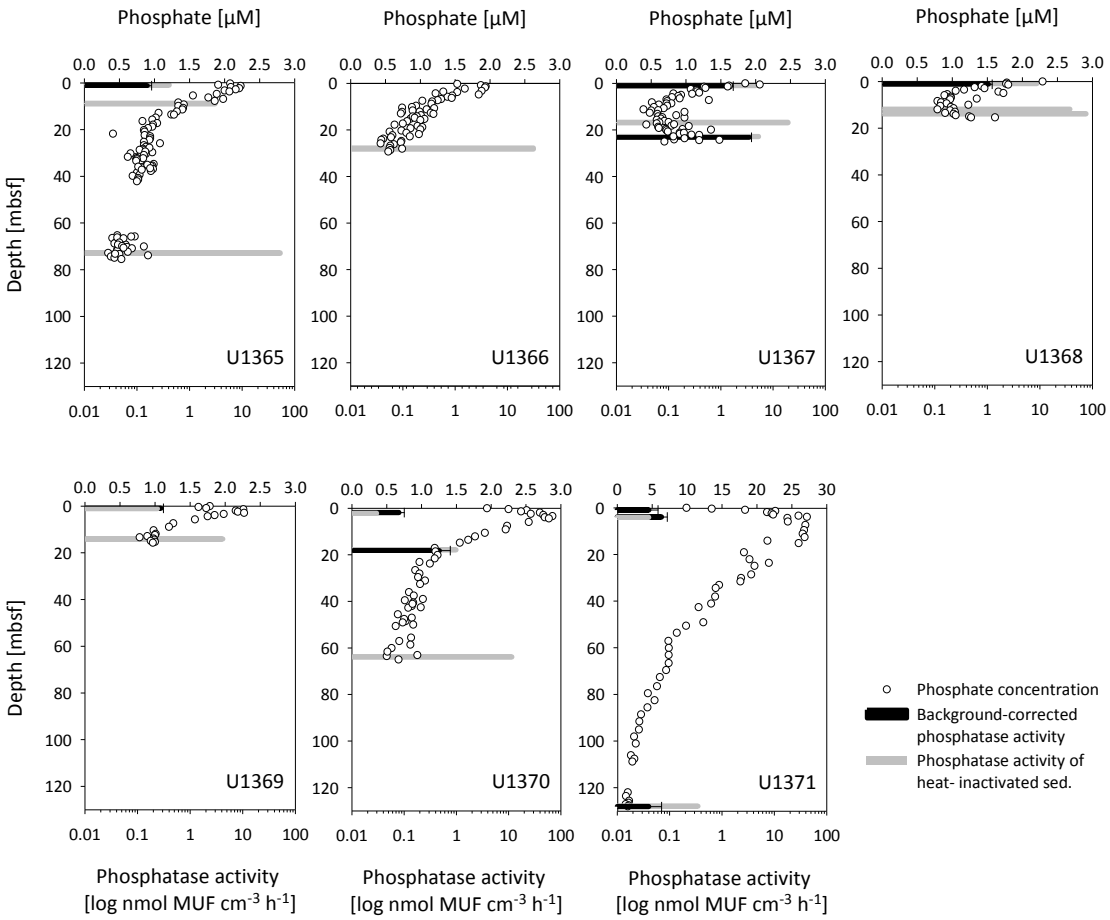

Figure 3: Comparison of phosphate concentrations and phosphatase activities at the seven South Pacific Gyre sites. Phosphate concentrations derive from ship-board data, available at the IODP Laboratory information management system (LIMS) reports database (web.iodp.tamu.edu).

The cell-specific phosphatase activities (0.15–866 pmol cell^{-1} h^{-1}) differed by three orders of magnitude (Figure 2c). The overall pattern followed that of the phosphate conversion of the untreated sediments. However, the difference found for the phosphatase activity between the surface samples and deeper layers was much more pronounced in the cell-specific calculations.

Aminopeptidase activities decreased with sediment depth

Most aminopeptidase activities were much lower than the phosphatase activities (Figure 2c, d). A low average background value of 0.005 nmol cm^{-3} h^{-1} was determined for the unspecific cleavage of the peptide bonds, (Figure 2d), indicating a successful heat inactivation of the control. One exception was Site U1366, where the measured background activity was 1.5 orders of magnitude higher than the average.

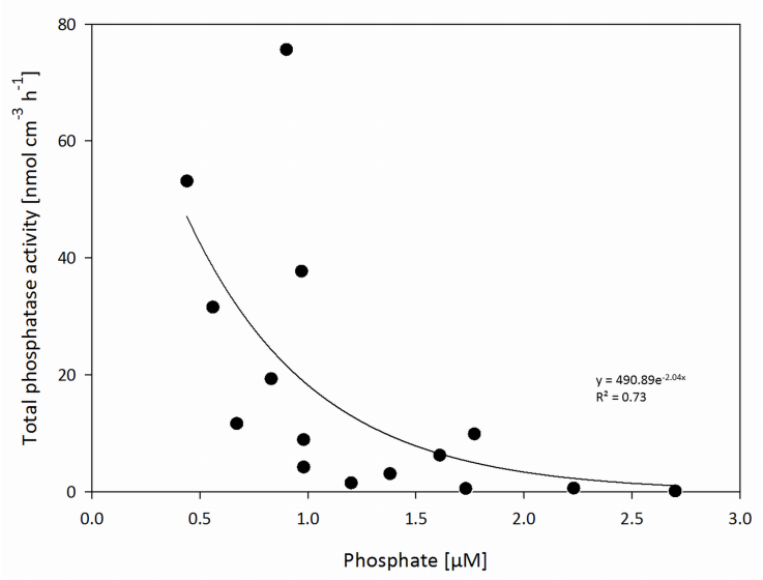

Figure 4: Correlation of total phosphatase activities and phosphate concentrations of the respective SPG sediment horizons.

Other than for the corrected phosphatase activity measurements, the aminopeptidase activities always exceeded the background rates (Figure 2d). Similar to the phosphatase activities, no trends along the two transects were observed, but a general decrease with sediment depth was found. While the average background-corrected aminopeptidase activities at surface-near layers were 0.093 nmol cm^{-3} h^{-1}, an average of 0.008 nmol cm^{-3} h^{-1} was found for the deepest layers. In contrast, the cell-specific activities of aminopeptidase showed an inverse trend with depth compared to the background-corrected aminopeptidase activities (Figure 2f). The span between the two end members, the surface of Site U1365 at the edge of the gyre and the bottom layer of Site U1367 close to the center of the gyre, increased from 0.009 to 27.5 fmol $cell^{-1}$ h^{-1}.

Pure cultures of the SPG exhibited exoenzyme activities

To investigate whether bacterial isolates from SPG sediments also exhibit exoenzyme activities, four pure cultures were tested exemplarily (Table 1). After microscopic screening of a total of 224 enrichments of which half the cultures revealed growth, the chosen strains derived from oxic enrichments containing 36 different monomeric carbon sources. These enrichments were inoculated with sediment slurries from 1.3, 122 and 128 mbsf, respectively of the outermost Site of the SPG (U1371; Figure 1). The

isolates were identified as relatives of *Halomonas* sp., *Erythrobacter* sp., *Nocardioides* sp. and *Dietzia* sp., all with 16S rRNA similarities of 99%. The presence of these isolates in the initial enrichments was already detected by molecular screening using denaturing gradient gel electrophoresis (DGGE). For the four investigated strains, the cell-specific aminopeptidase activities were 0.05-0.39 amol cell^{-1} h^{-1}. The phosphatase activities differed by a factor of ~17, ranging between 0.04 and 0.77 amol cell^{-1} h^{-1} (Table 1). While *Erythrobacter* sp. showed the highest phosphatase activity, *Halomonas* sp. revealed the highest aminopeptidase activity.

Table 1. Cell-specific exoenzyme activities of bacterial strains isolated from SPG sediments (IODP Site U1371).

Strain	Origin (mbsf)	Closest relative in GenBank	16S rRNA gene similarity (%)	Phosphatase activity (amol cell^{-1} h^{-1})	Aminopeptidase activity (amol cell^{-1} h^{-1})
FP001	128	*Halomonas* sp.	99	0.51 ± 0.032	0.39 ± 0.014
FP002	122	*Erythrobacter* sp.	99	0.77 ± 0.011	0.26 ± 0.004
FP003	1.3	*Nocardioides* sp.	99	0.04 ± 0.006	0.05 ± 0.007
FP004	122	*Dietzia* sp.	99	0.26 ± 0.034	0.31 ± 0.023

Discussion

Aminopeptidase activities generally decreased with depth which points to diminished microbial activities due to severe energy limitation in deeper, more oligotrophic layers. Interestingly, enhanced phosphatase activities were observed in layers with low phosphate concentrations. Although the microbial communities are probably not phosphate-limited, the measured activity might be an active response as retaining the enzyme activity over geological timescales is rather unlikely. The difference between surface and bottom layers for both cell-specific activities is probably due to the extremely low cell numbers counted for the SPG sediments.

Aminopeptidase activities reflect diminished microbial activities with sediment depth

Microbial communities in SPG sediments showed less aminopeptidase activities with depth, but still measurable amounts. The presence of aminopeptidase activities in SPG

sediments and all isolates support our initial assumption that virus particles might serve as a food source for starving microbial communities. The decreasing activities with sediment depth, in turn, would explain the increasing in virus-to cell ratios in deeper layers (Engelhardt *et al*., 2014). Low aminopeptidase activities probably result in a better conservation of virus capsids due to diminished protein degradation.

Low values for aminopeptidase activities in heat-inactivated samples allowed analyzing most sediment layers including a background correction. On the other hand, using the published assay with MCA as a fluorophore instead of MUF (Hoppe, 1993; Coolen & Overmann, 2000) resulted in a comparatively high autofluorescence of the sediments and thus diminished sensitivity. The procedure contains an acetone treatment of the samples after incubation which probably has mobilized absorbed organic compounds. For future studies on organic-poor sediments, it should be tested to return to the original protocol by Hoppe (1983) using MUF-Leu as substrate analogue to avoid any organic solvent as stop solution.

Are the measured phosphatase activities in SPG sediments due to conservation or active production?

The finding that decreasing phosphate concentrations with sediment depth resulted in increasing phosphatase activities is in accordance with previous studies on bacterial pure cultures and biofilms which have shown that the measured activities of phosphatases increased during phosphate starvation (Huang *et al*., 1998; Sebastian & Ammerman, 2009).

For the SPG sediments, the increasing activities are quite surprising, as indigenous microbial communities are probably not facing phosphate starvation. The lowest phosphate concentrations of 0.34 µM at 73 mbsf of Site U1365 would still be sufficient to support 3.6 x 10^3 cells cm^{-3} counted for this layer. Thus, the question arises why the cells invest the energy to produce exoenzymes especially when the background phosphatase activity is as high as determined for the SPG sediments. We can exclude that the relatively high background of phosphatase activities were caused by autofluorescence of the sediments. Boiling of the samples only enhanced the autofluorescence by less than five times which was also found for other sediments before (Belanger *et al*., 1997). The generally low autofluorescence reflects the low

organic carbon content of the SPG sediments which does not interfere with the phosphatase activity measurements as in other studies e.g. on Baltic Sea sediments (Steenbergh *et al*., 2011) or Mediterranean sapropels (Coolen & Overmann, 2000). Thermostability of immobilized phosphatases, in turn, would lead to an overestimation of the background activities. Nannipieri *et al*. (1982) e.g. could still detect activities of phosphatases when the enzymes were attached to soil particles after incubation at 80 °C for 2 hours. However, for substrate uptake by indigenous microorganisms, the source of bioavailable phosphate does not make a difference.

The cost-intensive production and release of exoenzymes is energetically unfavorable and would indicate preservation rather than active production. Unfortunately, the long-term stability of exoenzymes in marine sediments was not investigated so far. On the other hand, the correlation of phosphatase activities to phosphate concentrations suggests an active process, as retaining the enzyme activity over geological timescales is rather unlikely. Furthermore, if the activity would only be caused by conservation over time and depth, the recharge of phosphate at the bottom layer of Site U1367 by crustal-fluid diffusion would not diminish phosphatase activities. An active process is also suggested by the phosphatase activities exhibited by the isolates tested. The varying phosphatase activities of the isolates implement different ecological strategies. Relatives of *Erythrobacter* sp. probably contribute to the pool of phosphatases within the sediment layers they were isolated from, as indicated by highest cell-specific phosphatase activities. Relatives of *Nocardioides* sp., in turn, only release minor amounts of phosphatases and aminopeptidases. Thus, these microorganisms might take advantage of the activity of other microbial community members.

The exoenzyme activities in SPG sediments are not exceptionally low

As we expected to find very low exoenzyme activities within the SPG sediments that exhibit extremely low cell numbers and oxygen respiration rates (D'Hondt *et al*., 2015), we applied relatively high substrate concentrations and incubations at room temperature. Since both factors positively influence exoenzyme activities, high concentrations of substrates are suggested to be used to determine relative exoenzyme activities (Hoppe, 1993). As most of the published experiments were

performed under in-situ temperatures and/or low substrate concentrations (Boetius, 1995; Boetius *et al*., 1996; Fabiano & Danovaro, 1998; Coolen & Overmann, 2000), they are not directly comparable with our results. Only two studies were performed on sites that exhibit comparable trophic conditions, sediment depth and age (Table 2). For deep-subsurface sediments of the Juan de Fuca Ridge (Engelen *et al*., 2008), the highest phosphatase activities were comparable to our lowest measurements. For Mediterranean clay (Coolen & Overmann, 2000), our results were lower or similar to the exoenzyme activities determined. The authors interpreted the measured exoenzyme activity as an indicator of metabolically active bacteria.

Table 2. Phosphatase- and aminopeptidase activities within marine sediments. Ranges represent different sites and sediment horizons.

Origin	**Sed.-depth**	**Phosphatase activity**		**Aminopeptidase activity**		**Ref.**
(mbsl)	**(mbsf)**	**(nmol cm^{-3} h^{-1})**	**(fmol $cell^{-1}$ h^{-1})**	**(nmol cm^{-3} h^{-1})**	**(fmol $cell^{-1}$ h^{-1})**	
SPG (3740 - 5695)	0.9-128	0.04–3.6	0.15-866	0.0002–0.4	0.009-28	This study
Mediterranean clay (2150 - 3515)	0-3.60	0.5-3	0.01-0.05	0.02–2.5	0.005-0.02	(1)
Hidaka Trough (1200)	0-365	+	n.d.	+	n.d.	(2)
Juan de Fuca Ridge (2660)	0-260	0.003–0.045	n.d.	n.d.	n.d.	(3)$
North Atlantic (4500)	0-0.1	n.d.	n.d.	5–14.7	2.9	(4)*
Mediterranean sea (897 - 4260)	0-0.01	n.d.	n.d.	14-284	0.03-0.6	(5)*
Ross Sea (439 - 567)	0-0.15	n.d.	n.d.	1.3-2.7	0.001-0.003	(6)*
Mediterranean sapropels (550 - 2150)	0.25-4.9	2-8	0.005-0-06	0.8–1.8	0.0008-0.03	(1)
German tidal-flat (0 - 1)	0.035-0.11	12-142	0.001-0.14	6-32	0.001-0.03	(1)*

n.d. = not determined, + = activity detected, * = numbers recalculated from published data, $ = after recalculation of raw data, References: 1 (Coolen & Overmann, 2000), 2 (Kobayashi *et al*., 2008), 3 (Engelen *et al*., 2008), 4 (Boetius, 1995), 5 (Boetius *et al*., 1996), 6 (Fabiano & Danovaro, 1998)

Based on published average turnover numbers of phosphatases and aminopeptidases for bacteria (Schomburg *et al*., 2004), we calculated that the measured enzyme activities would imply an amount of 0.16-15 and 0.03-73 pg of enzymes cm^{-3}, respectively. These numbers are equal to 2 x 10^5 to 4 x 10^8 enzyme molecules cm^{-3}. As we have measured potential exoenzyme activities under elevated temperature and substrate concentrations, we assume the in-situ rates to be even lower. Comparing our data with normalized cell-specific exoenzyme activities of other marine sediments is influenced by the extremely low cell numbers determined for the SPG (Kallmeyer *et al*., 2012). Minor counting errors would result in large deviations and consequently in overestimations of the cell-specific activities (D'Hondt *et al*., 2015).

Author contributions

FP: Isolation of pure cultures, activity measurements, data analyses (calculations, creation of tables and figures), data interpretation, first draft

HC: Data analyses (calculations and interpretation), revision of the manuscript

BE: Idea and concept, pre-experiments, data interpretation, writing and revision

Acknowledgments

The authors are grateful to Anja Grubert, Julius Degenhardt and Dennis Tebbe for pre-experiments on exoenzyme activity measurements with bacterial isolates. This research used samples provided by the Integrated Ocean Drilling Program (IODP). We would therefore like to thank the IODP Exp. 329 science party and especially Tim Engelhardt for setting up initial enrichment cultures. The work was financially supported by the German Research Foundation (DFG).

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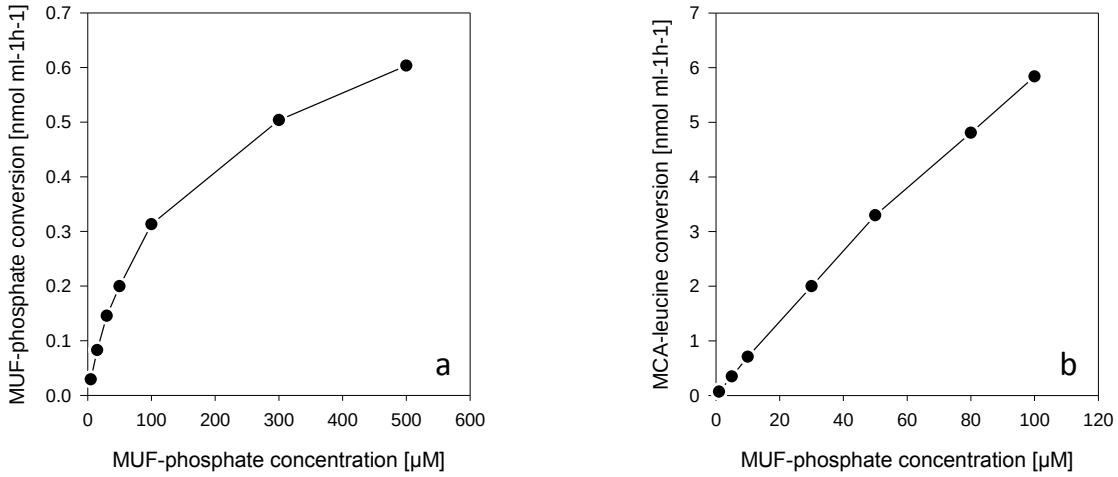
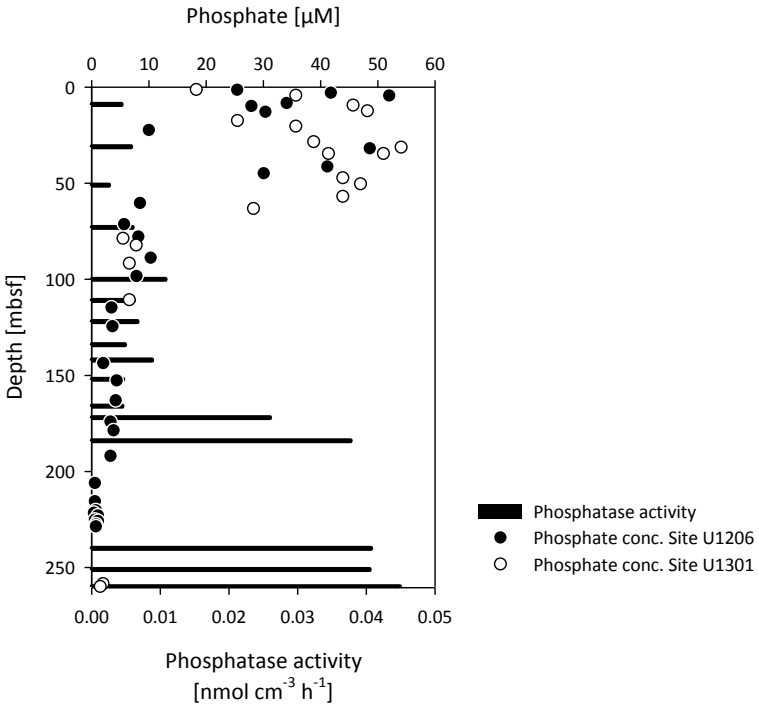
Supplementary Material



Supplementary Figure 1. Phosphatase- (a) and aminopeptidase (b) activities at different substrate concentrations measured with a pure culture of *Erythrobacter* sp.



Supplementary Figure 2. Comparison of phosphatase concentrations and phosphatase activities at IODP Site U1301. Background-corrected phosphatase activities were newly processed from raw data (Engelen *et al.,* 2008) to eliminate a calculation error. Phosphate concentrations from the nearby IODP Site U1026 were plotted additionally to fill up missing measurements in the depth profile of IODP Site U1301.

2.3. Microbial communities in extremely oligotrophic sediments of the South Pacific Gyre: Do they feed on phages?

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To be submitted

Abstract

Microbial communities in extremely oligotrophic sediments of the South Pacific Gyre (SPG) face severe electron-donor limitation. Thus, they need to develop strategies for the acquisition of degradable organic matter. Virus particles have been reported to potentially exceed bacterial biomass in deeper sediment layers of the SPG. As these particles are mainly built of nucleic acids and proteins, it is assumed that they might represent a food source for indigenous microorganisms. In this study, we have incubated bacterial strains that were isolated from SPG sediments as well as SPG bulk-sediments with concentrated Rhizobiophages from *Rhizobium radiobacter* strain P007 as sole carbon source. Changes in numbers of virus particles and bacteria were followed by direct counting and quantitative PCR (qPCR) of the antirepressor gene of the Rhizobiophages during the course of the experiments. We expected to observe a simultaneous reduction of viral particles and an increase in bacterial biomass. Decreasing viral counts were detected by both measurements within the cultures comprising bacterial isolates and concentrated phage particles. Moreover, decrease of virus particles due to viral decay could be excluded by control cultures that did not contain bacterial isolates. Similar results as for the bacterial cultures were found for the experiments with bulk sediments of the SPG. However, the decrease of phage particles was much more pronounced in the qPCR that specifically targeted the Rhizobiophages. By direct counting, quantification was biased by recording of all viruses present in the cultures, including spontaneously induced phages of the strains. The observed increase in bacterial counts, however, can not only be explained by feeding on phages as HPLC measurements of the Rhizobiophage concentrates revealed the presence of glycerol which might have served as an additional carbon source. This substance was transferred into the experiment during the phage concentration procedure using spin columns. Consequently, the increasing bacterial biomass was probably due to co-feeding on phages and glycerol. In conclusion, the decrease in virus numbers is still a strong indicator that supports our initial assumption that phage particles might serve as a food source for deep subsurface microorganisms.

Introduction

Growth and activity of prokaryotic communities in subsurface sediments are strongly dependent on the availability of organic matter and suitable electron acceptors (Canfield *et al*., 1993; Jørgensen, 2000; DeLong, 2004). The amount and quality of the material that reaches the seafloor, in turn, is related to the primary production within the overlying water column and the water depth (Engelen & Cypionka, 2009). Continental margins are generally characterized by high prokaryotic numbers and fast remineralization of organic matter due to an input of nutrients from the landsite and a relatively shallow water depth (Poremba *et al*., 1999; Beck *et al*., 2009). This is reflected by oxygen-depletion within the first millimeters to centimeters of the underlying sediments and steep geochemical gradients within the sediment column (Llobet-Brossa *et al*., 2002; Billerbeck *et al*., 2006; Teske & Sorensen, 2008). With increasing distance from the continents, the open ocean becomes more oligotrophic and is characterized by diminished input of fresh organic matter, which leads to a reduced primary production (Jahnke, 1996). As the degradation process already starts during sedimentation, consequently, only low amounts of highly recalcitrant material reach the seafloor (Suess, 1980; Engelen & Cypionka, 2009). The most oligotrophic marine provinces are the mid-ocean gyres which cover approximately 48% of the world´s oceans (D'Hondt *et al*., 2009). The largest of these gyres, the South Pacific Gyre (SPG), was investigated during Expedition 329 of the International Ocean Discovery Program (IODP) in 2010 (D´Hondt *et al*., 2010). Low sedimentation rates between 0.008 and 1.1 mm kyr-1 (D'Hondt *et al*., 2009) lead to diminished oxygen consumption by aerobic respiration (Fischer *et al*., 2009). Kallmeyer *et al*. (2012) reported cell counts for the SPG sediments that are orders of magnitude lower than in sediments of ocean margins and upwelling regions with equivalent water depth. Accordingly, oxygen and nitrogen are not depleted and penetrate deep into the sediment column (D´Hondt *et al*., 2011; D'Hondt *et al*., 2015), resulting in oxygenated sediments even down to the basaltic crust (Fischer *et al*., 2009; Røy *et al*., 2012). As a consequence, microorganisms in SPG sediments are not limited by electron-acceptor availability, but face severe energy limitation due to a lack of electron donors (D'Hondt *et al*., 2015).

Interestingly, in a study by Engelhardt *et al*. (2014) increasing virus-to-cell ratios with sediment depth were reported for SPG sediments, indicating that the viral biomass in

deeper sediment layers might exceed the prokaryotic biomass. Virus particles mainly consist of nucleic acids surrounded by protein capsids (Weinbauer, 2004), which might serve as an easily degradable substrate for indigenous microorganisms. To degrade larger polymers into smaller building blocks, bacteria are able to produce several types of exoenzymes (Weiss *et al*., 1991). In a recent study, we tested whether SPG sediments exhibit exoenzyme activities (Preuss *et al*., submitted). Aminopeptidase activity as a measure of protein degradation was detected in most of the investigated layers, but decreased with depth according to diminished microbial activities. Additionally, it was shown that pure cultures isolated from SPG sediments were also able to produce aminopeptidases. These results imply that the preconditions for potential virus degradation in the SPG are given. Thus, the question arises whether indigenous microbial communities in SPG sediments might take advantage of utilizing phage particles as food sources.

To test this hypothesis, a mixture of four SPG isolates was cultivated in artificial seawater medium amended with concentrated Rhizobiophages. Rhizobiophages are inducible prophages from the Alphaproteobacterium *Rhizobium radiobacter* which represents a widely distributed and the most often isolated bacterium from subsurface sediments (Süß *et al*., 2006; Engelhardt *et al*., 2011). For this study, *R. radiobacter* strain P007 was chosen due to the availability of specific primers for the detection of the inducible prophages (Engelhardt *et al*., 2013). During the course of the experiment, quantification of virus particles and bacteria was performed by direct counting and quantitative PCR. While direct counting included all viruses present in the cultures, quantitative PCR specifically targeted the antirepressor-gene of the Rhizobiophages. A similar feeding experiment with sediment slurries from IODP Site U 1367 was performed to test if the results from the pure cultures could be verified by incubating bulk sediments amended with Rhizobiophages.

Methods

Preparation of Rhizobiophage concentrates

For the production of Rhizobiophages from *Rhizobium radiobacter* strain P007 an induction experiment using mitomycin C as inducing agent was performed according to the protocol of Chen *et al.* (2006). *Rhizobium radiobacter* strain P007 was grown in

artificial seawater medium after Süß *et al.* (2004) with 10 mM glucose at 20 °C and a shaking speed of 100 rpm. The initial culture was split into two subcultures when reaching the exponential phase, measured at an optical density of 600 nm (OD_{600}). One subculture was treated with mitomycin C (1 µg ml^{-1} w/v, final concentration) for 30 min, while the second served as control. After the incubation, both cultures were washed twice by centrifugation for 20 min at 20 °C and 7500 rpm (Beckmann-Centrifuge J2-HS, Rotor JA-10) and resuspended in fresh artificial seawater medium. The cultures were further incubated until the OD_{600} of the induced culture decreased as an indication of phage induction. After that, the culture was centrifuged as described above to remove the cells. For further removal of remaining bacterial cells and particles, the supernatant was sterile filtered three times, first with a 0.45 µm pore-sized cellulose nitrate filter, followed by 0.2 µm pore-sized cellulose acetate filter (Sartorius stedium biotech GmbH, Göttingen, Germany) and finally by a 0.1 µm pore-sized disposable filter (Whatman®, GmbH Dassel, Germany). Purified Rhizobiophages were concentrated using Vivaspin 20 centrifugation tubes, which contained a 100 kDa pore-sized membrane (Sartorius stedium biotech GmbH, Göttingen, Germany). Rhizobiophage concentrates were stored at -70 °C until further processing. Potential cross-infections of SPG isolates by Rhizobiophages were excluded with Plaque assays for each strain.

Feeding experiments on Rhizobiophages by isolates of the South Pacific Gyre

In order to test putative utilization of phage particles by indigenous bacteria of SPG sediments, pure cultures of *Halomonas* sp., *Erythrobacter* sp., *Nocardioides* sp. and *Dietzia* sp. (Preuss *et al*., submitted) were pre-cultured in artificial seawater supplemented with 10 mM glucose and incubated for at least one month. In total, three different cultures with a volume of 50 ml each were set up. The main culture consisted of artificial seawater (without glucose), concentrated Rhizobiophages (titer: 9.5 x 10^{10} virus-like particles ml^{-1}) and a mixture of SPG isolates. Furthermore, two cultures served as control. For the detection of viral decay, the cell-free control consisted of artificial seawater and concentrated Rhizobiophages. To observe any self-induction of prophages from the SPG strains, the phage-free control only contained artificial seawater and the SPG isolates.

Two separate feeding-experiments with SPG cultures were performed. The first experiment was conducted to test the general applicability of the method. All three setups were incubated for seven days. A total of 11 subsamples were taken regularly after 0, 3, 6, 9, 12, 24 h at the first day and afterwards once every day. Subsamples for virus counts were filtered through 0.1 µm pore-sized disposable-filters (Whatman®, GmbH Dassel, Germany) and frozen at -20 °C until further processing. Subsamples for quantitative PCR (qPCR) were frozen directly after sampling. The second experiment served as a repetition for the first experiment. During 14 days of incubation, subsamples for virus and additonally bacteria counts as well as for qPCR were initially taken every 12 hours and afterwards on a regularly basis. Subsamples for virus counts and qPCR were treated as described above, those for bacteria counts were fixed with formaldehyde (2%, final concentration). All subsamples were stored at -20 °C until further processing.

Feeding experiments on Rhizobiophages by an indigenous microbial community within South Pacific Gyre sediment

Putative feeding on Rhizobiophages was exemplarily tested on near-surface sediment (1.2 mbsf) from the SPG (IODP Site U1367). First, 4 cm^3 of sediment were diluted in a total volume of 40 ml artificial seawater medium (1:10 dilution). Again, three cultures were setup, containing the main culture with concentrated phages, a phage-free and a cell-free control. For the cell-free control, the sediment slurry was autoclaved three times for 20 min at 121 °C interrupted by incubations over night at 20 °C. For each culture, 4 ml of the slurry were transferred into sterile glass bottles. 8 ml of phage concentrate (1.05 x 10^9 virus-like particles ml^{-1} final concentration) were added to the main culture as well as to the cell-free control. The cultures were incubated at 20 °C for 68 days. A total of ten subsamples were taken for qPCR analysis (0.5 ml in duplicates) and stored at -70 °C until further processing.

Direct counting of virus-like particles (VLP) by epifluorescence microscopy

Subsamples for virus counting were filtered onto 0.02 µm Anodisc filters (13 mm, Whatman, England) and washed with TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The filters were mounted onto a microscopic slide using 3-4 µl of Moviol solution (Fluka,

Switzerland). VLP were stained with SYBR-Green I (Life technologies TM) and a minimum of 400 VLP within 10 to 20 randomly chosen fields were counted with an epifluorescence microscope (Olympus BX51TF, Japan; 600-fold magnification).

Quantification of Rhizobiophage- and 16S rRNA-templates by qPCR

The antirepressor gene of RR1-A of the Rhizobiophage 1-A (Engelhardt *et al.*, 2013) and 16S rRNA genes of the SPG isolates and indigenous bacteria from SPG sediments were quantified by using specific primer pairs. The pair RR1-A-F and RR1-A-R (fragment length: 624 bp) was used for amplification of the phage antirepressor gene in order to generate qPCR standards. PCR was performed as described by Engelhardt *et al*. (2013)and Süß *et al*. (2004) with a few adjustments. Instead of 30 cycles, a total of 35 cycles was applied and the annealing time for the amplification of the almost complete 16S rRNA genes was 42 °C. The amplicons were further purified (Qiagen PCR Purification Kit, Qiagen) and the DNA content was determined with a NanoDrop 2000c Spectrophotometer (Thermo Scientific). The number of gene targets was calculated from the respective length of the DNA fragment, the amount of DNA and the average weight of base pairs (1.1×10^{-21} g). A standard curve for the RR1-A gene was prepared by a 10-fold dilution in a range between 10^0 and 10^9. Quantification of RR1-A targets was done according to Engelhardt *et al*. (2013) with a few adjustments using a second primer pair RR1-As-F and RR1-As-R (fragment length: 124 bp). We used the 2 x DYNAmo HS, F410 mastermix (Thermo Scientific) and applied a total of 50 cycles. Collected data was analyzed using the Rotor-Gene analysis software V. 4.6.94. Quantification of bacterial 16S rRNA gene fragments was performed as described above but by using the primer pair 8f/1492r for generating qPCR standards and the pair 357f/907r (Lane, 1991) for quantification with annealing temperatures of 56 and 57° C, respectively.

DNA-extraction from sediment slurries

Prior to enumeration via qPCR, the PowerSoil® DNA Isolation kit (MoBio) was used for the sediment slurries according to the manufacturer´s instructions with a few adjustments: 0.5 ml of the slurries was used for DNA extraction and the DNA was eluted in 50 µl PCR-grade water. The DNA amount (in ng/ µl) as well as the purity of the

samples was measured with a NanoDrop 2000c Spectrophotometer (Thermo Scientific). Samples were stored at – 20 °C until further processing.

HPLC analysis

In order to exclude the transfer of glucose from the initial cultures into the feeding experiments via the phage concentrates, HPLC analysis was performed using a SYKAM system (SYKAM GmbH) with a linear UVIS 204 (UV/ Vis) detector and a refractive index (RI) detector. All tested samples were filtered (13 mm Syringe filter, 0.2 µl PTFE membrane, VWR™ international USA) in 1.5 ml glass vials and measured in the UV/ Vis detector at a wavelength of 210 nm and in the RI detector at ambient temperature. Analysis of data was performed with the ChromStar DAD 6.3 software.

Results

Direct counts and qPCR measurements showed similar trends

The direct counts of VLP of the main culture that contained the SPG isolates and concentrated Rhizobiophages decreased over the seven days of incubation (Figure 1a).

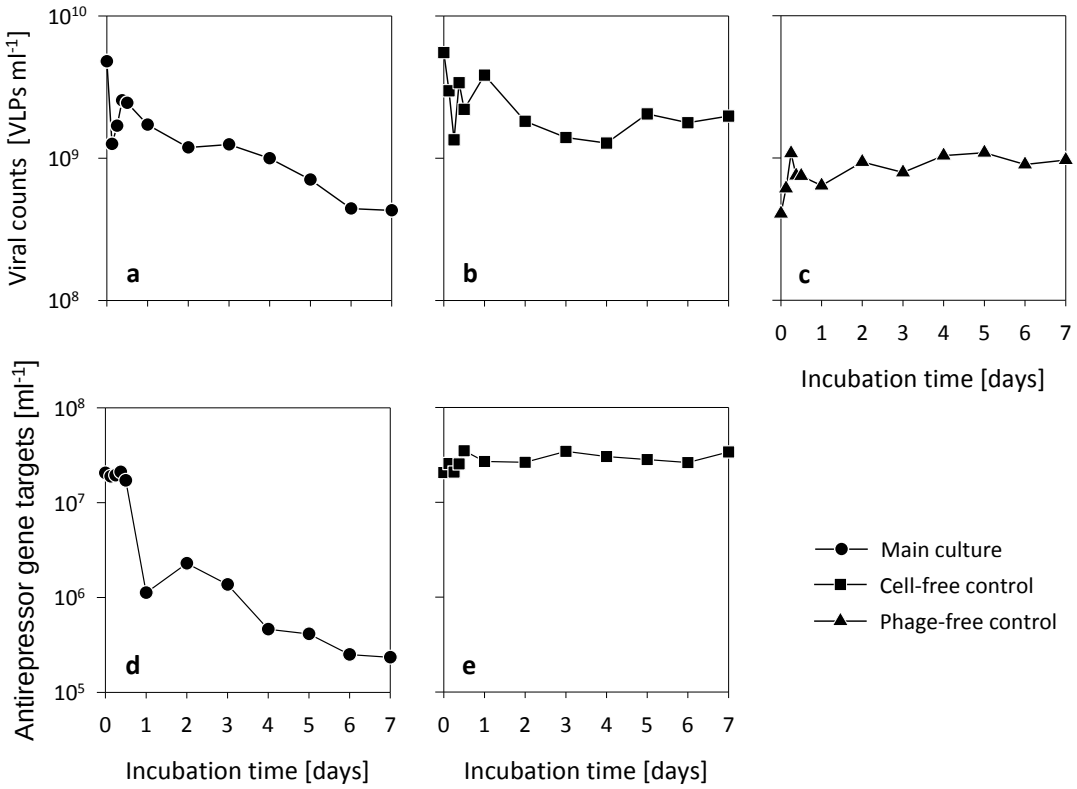

Figure 1. Virus counts (a-c) determined by direct counting and quantification of phage antirepressor genes (d, e) of viruses after 7 days of feeding SPG isolates with Rhizobiophages.

Starting and end values differed by about one order of magnitude (4.8 x 10^9 and 4.3 x 10^8 VLP ml^{-1}). In contrast, the viral decay in the cell-free control accounted for only half an order of magnitude (Figure 1b) and fluctuated strongly within the first day. Viral numbers in the phage-free control that resulted from spontaneously induced prophages from the isolates changed only slightly within the first hours of incubation, but stayed on a constant level until the end of the experiment (Figure 1c). Quantification of the Rhizobiophage antirepressor-gene by qPCR revealed a similar, but even stronger decrease with time of 2.1 x 10^7 and 2.3 x 10^5 gene targets ml^{-1} (Figure 1d). No changes were measured by qPCR for the cell-free control (Figure 1e).

Bacteria counts increase simultaneously to decreasing virus counts

Quantification of the Rhizobiophage antirepressor gene from the second feeding experiment with the SPG isolates showed a decrease of Rhizobiophage particles of about two orders of magnitude. While the trend for the main culture was not as clear, it was still similar to that of the first experiment (Figure 1a and 2a). The antirepressor-gene targets of the cell-free control decreased within the first eight days indicating a viral decay, but increased afterwards to reach the starting value at the end of the experiment (Figure 2b). However, the values of the control were always higher than the respective measurements of the main culture.

In general, the 16S rRNA gene targets within the main culture increased slightly from 1.9 to 3.8 x 10^8 targets ml^{-1} within the first 6 days, but decreased afterwards by almost 6-fold to an end value of 6.6 x 10^7 targets ml^{-1} (Figure 2c). Although the phage-free control was setup from the same pre-culture and with the same amount, the starting concentration of 16S rRNA genes was about one order of magnitude lower than that of the main culture (Figure 2d). Over the whole time of incubation the number of 16S rRNA genes increased about 2-fold.

Phage concentrates for the feeding experiments were found to contain glycerol

The purity of the Rhizobiophage concentrates was analyzed with regard to a putative transfer of glucose from the induction experiment with *R. radiobacter*. Glucose was not detected, but HPLC analysis revealed the presence of an unknown byproduct in the RI detector after concentrating the Rhizobiophages which could serve as a potential food source (Supplementary Figure 1; highlighted with a blue star).

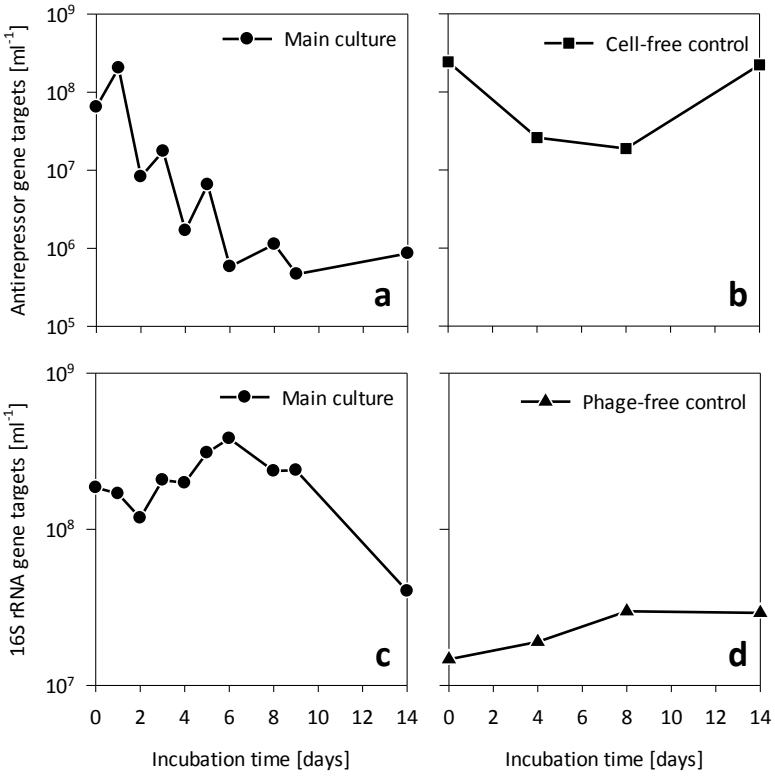

Figure 2. Counts of viruses (a,b) and bacteria (c,d) after 14 days feeding of SPG isolates on Rhizobiophages determined by qPCR. Main cultures (a,d) are shown by points, cell-free (b) and phage-free (d) controls are indicated by squares and triangles, respectively.

This contamination was probably released by the Vivaspin 20 centrifugation tubes (Sartorius) used for concentration of the phages as it was only detected in the concentrates and not in the initial phage lysates (Supplementary Figure 2, indicated by arrow). This substance was identified as glycerol with concentrations of 10-15 mM. Within the main culture of the feeding experiment with SPG isolates, an initial glycerol concentration of 5mM was detected, but was not measurable at the end of the experiment. Thus, glycerol was probably degraded by the isolates.

Feeding of microbial communities in SPG sediment slurries revealed ambiguous results

The development of viruses and bacteria in sediment slurries of Site U1367 were followed by qPCR over 68 days. In general, the antirepressor-genes of Rhizobiophages within the main culture increased slightly over the first three weeks and stagnated afterwards (Figure 3a). Bacterial 16S rRNA genes decreased within the first 7 days, followed by an increase until day 23 and then stagnated until the end of the

experiment (Figure 3c) This indicates an initial decline of the microbial community and subsequent growth afterwards. Numbers of the antirepressor-gene targets in the cell-free control strongly fluctuated, but were always lower than in the main culture (Figure 3b). However, comparing starting and end values, an increase by half an order of magnitude was determined. In contrast, 16S rRNA gene targets of the phage-free control decreased within the first 33 days by almost 2 orders of magnitude from 8.3×10^5 to 5.6×10^4 targets ml^{-1}, followed by an increase to 2.8×10^5 targets ml^{-1} (Figure 3d).

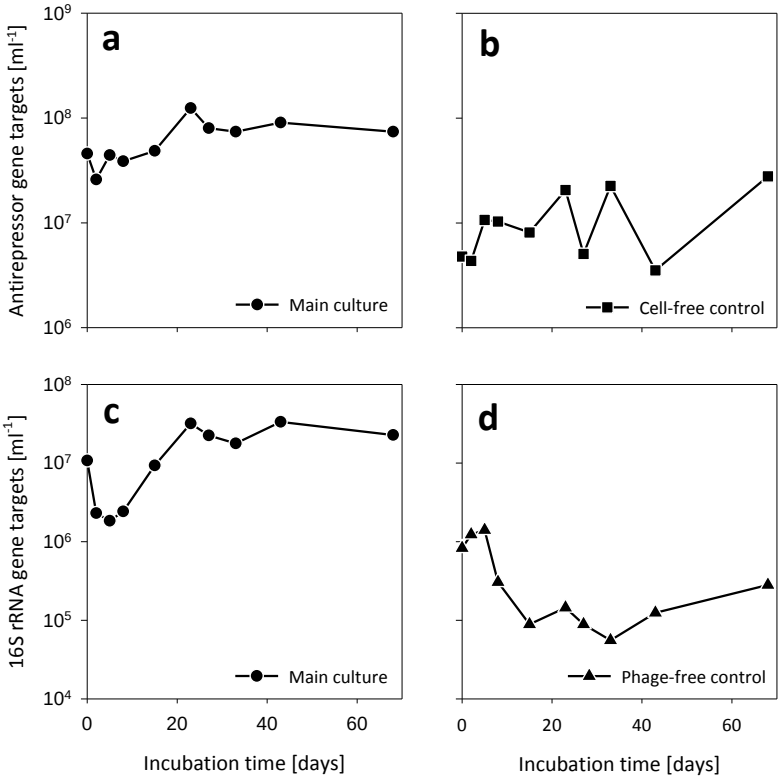

Figure 3. Antirepressor (a, b) and 16S rRNA (c, d) gene targets determined by qPCR after 68 days of feeding a microbial community of sediments from SPG Site U1367 with Rhizobiophages. Values of the main culture are shown in points (a, c). Cell-free (b) and phage-free (d) controls are indicated by squares and triangles, respectively.

Discussion and Outlook

Decreasing viral counts in comparison to the control measurements indicate utilization of Rhizobiophages by SPG isolates

Viral counts were measured by two different methods in this study. Determination by qPCR of both culture experiments revealed the same decreasing trend of Rhizobiophages. The degradation of viruses was additionally verified by the direct

counts. A decrease in viral counts due to viral decay can be excluded as the counts in the controls stayed either constant or decreased less than that of the main cultures. Consequently, both types of measurements can be assessed as useful methods for following viral counts in feeding experiments with pure cultures. Discrepancies in the absolute numbers between both methods are likely due to the fact that direct counting of viruses not only included the Rhizobiophages, but also spontaneously induced phages of the SPG isolates. These might either have been transferred from the pre-culture or have been produced during the experiment. Furthermore, only one of the two Rhizobiophages of *R. radiobacter*-strain P007 was targeted for detection by qPCR. Thus, differences in the degradation patterns might also result from the methods themselves.

Final proof of phage utilization via increase of bacterial biomass was biased by an additional food-source

In general, the increase in cell counts within the main culture determined by two different methods is an indicator for growth of the SPG isolates. As this growth coincided with the decrease of viral counts, our initial assumption of feeding on virus particles by the mixed culture of SPG isolates was supported. Calculations based on the determined phage titer and the added volume of the phage concentrates in combination with an estimated biomass of 0.2 fg per virus (Suttle, 2005) showed that approximately 2.84 x 10^{11} fg carbon were provided as a putative food source to the SPG isolates. At least one and a half doublings of the original cells would have been possible, assuming an average amount of 14 fg carbon per cell in sub-seafloor environments (Kallmeyer *et al*., 2012) and an assimilation rate of 60% together with the cell counts.

Unfortunately, an increase in cell counts was also detected in the phage-free control and the cell counts of the main culture were slightly higher than what would have been assumed from the calculations. Reasons could be e.g. a more effective assimilation of the phage particles, storage compounds of the added cells or the utilization of inducible phages from the other strains of the SPG culture mix. However, as indicated by the phage-free control as well as the detected transfer of glycerol from the phage concentration procedure, growth of the bacterial isolates was likely not only due to

feeding on phage particles. It might rather have been a result of degrading the glycerol in combination with the phages as a potential co-substrate. However, the extracellular degradation of the phage particles and import of amino acids might be less cost intensive than building biomass from glycerol. Moreover, the combined utilization of both substrates is inferred by the detected differences between the main culture and the phage-free control.

For further experiments, prewashing steps prior to the concentration of phages are recommended to reduce or totally wash out the glycerol. Direct tracing of phage-incorporation could additionally be performed by growing the *R. radiobacter*-culture with ^{13}C-Glucose during the induction experiment and thus, harvesting labeled phages. During pre-experiments using labeled phages (data not shown) several unidentified substances were detected as impurities of the ^{13}C labeled glucose and products deriving from the induction experiment. Thus, it could not be excluded that these compounds also contained the ^{13}C-label. An incorporation of these compounds into the biomass of the SPG isolates would also interfere with the proof that the labeled phages were used as a food source. Consequently, also this method needs further development.

Feeding experiment on SPG sediments

Increasing antirepressor-gene targets within the SPG sediment slurries of both, the main and the cell-free culture in the beginning of the experiment are rather hard to explain. This finding points towards the presence of *R. radiobacter* in the sediments or a transfer through the concentrated Rhizobiophages from the induction experiment. However, running a *R. radiobacter*-specific PCR with DNA extracted from the SPG sediment revealed the absence of this species. Additionally, no bacterial growth was observed on agar plates that were incubated with the phage concentrates (data not shown). Thus, the increase in antirepressor gene targets within both cultures cannot finally be explained. Moreover, it has to be taken into account that besides the added Rhizobiophages also a number of viruses were already present in the sediment used for this experiment. Calculation of the ratio, however, showed that the added Rhizobiophages exceeded the indigenous viral community by a factor of 9000. As the microbial community present in the sediment slurry does likely not distinguish

between Rhizobio- and indigenous phages, the expected decrease in counts of the antirepressor-gene targets would probably not be as steep as with the culture experiments.

The bacterial counts within the main culture, in turn, indicated an initial decay that could possibly be caused by stress due to the dilution and subsequent release of prophages from the lysed cells. The following growth phase might have been stimulated by feeding on (i) remaining cell debris from the initial lysis, (ii) the added Rhizobiophages and other phages present in the sediments or (iii) the glycerol.

The predators might become prey

In general, viruses are known to redirect the host cell metabolism for their own reproduction and to finally kill the host (Weinbauer, 2004). Therefore, they can be seen in the role of "predators" for their host. The results of our study support our initial hypothesis that in the deep-sea sediments of the SPG, the reverse might be true. Our interpretation that SPG isolates are feeding on phage particles mainly derived from the decrease in viral counts compared to the controls. In contrast, the visible increase in culture turbidity as well as increasing bacterial cell counts were probably due to a co-feeding on both, the phage particles and the glycerol.

Precondition for the utilization of polymeric substances is the degradation to smaller units by exoenzymes (Weiss *et al*., 1991). As virus particles mainly consist of a nucleic acid that is surrounded by proteins (Weinbauer, 2004), aminopeptidases would be required for the degradation of the protein capsid. In a pre-study it has already been shown that the four SPG isolates are able to produce aminopeptidases (Preuss *et al*., submitted). Additionally, it was tested whether SPG sediments exhibit exoenzyme activities. Aminopeptidase activity was detected in most of the investigated layers, indicating that the preconditions for potential virus degradation are given. Together with the reported increase in virus-to-cell ratio with sediment depth (Engelhardt *et al*., 2014), the required substrate would be available. Thus, due to the highly recalcitrant organic material in this part of the deep-biosphere, bacteria might have developed a strategy to utilize the remaining easily degradable food sources. However, phages alone will certainly not be the sole available food source for microbial communities in SPG sediments. As the lysogenic life cycle correlates to a low host density, it is assumed

to be the preferred proliferation mode of viruses in environments with low cell numbers (Fuhrman, 1999; Weinbauer, 2004; Danovaro *et al*., 2008). In contrast to the lytic life-cycle, this strategy does not destroy the host cell and viral reproduction occurs in parallel to cell division (Fuhrman & Suttle, 1993). Even though environmental conditions in the deep subsurface seems to be rather stable over geological time scales, stress factors for the cells e.g. changes in temperature, salt concentration or physical stress might reach a certain threshold and thus, induce the prophages to switch to the lytic life mode which would results in cell lysis (Paul, 2008). The remaining fragments of the host cells could contribute to the pool of particulate and dissolved organic matter (Fuhrman, 1999; Suttle, 2005) and might therefore represent an additional food source for indigenous microorganisms in deep-subsurface sediments of the SPG.

Author contribution

FP: Concept, Supervision of measurements, sampling, data analyses (calculations, creation of figures), data interpretation, first draft

LMM: Concept, Experimental procedure, sampling, measurements, data analysis (calculations), revision of manuscript

HC: Revision of the manuscript

BE: Idea and concept, data interpretation, writing and revision

Acknowledgements

The authors would like to thank Jana Feldkamp for the laboratory support. This research used samples provided by the Integrated Ocean Drilling Program (IODP). We would therefore like to thank the IODP Exp. 329 science party and especially Tim Engelhardt for the sampling procedure. The work was financially supported by the German Research Foundation (DFG).

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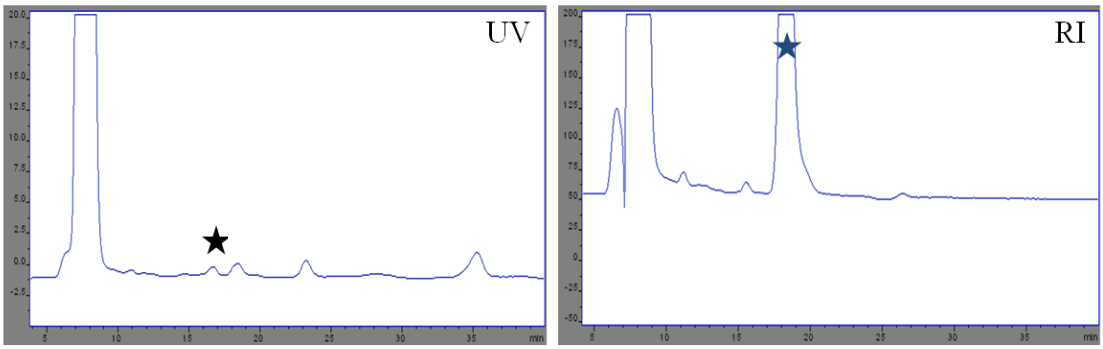
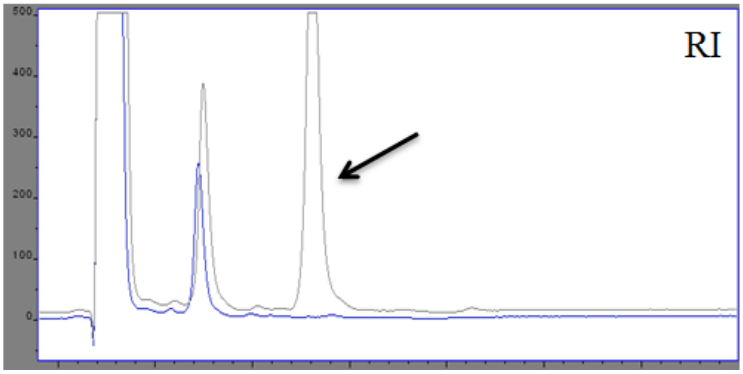
Supplementary Material



Supplementary Figure 1. HPLC chromatograms of concentrated Rhizobiophage lysates detected by an UV-Vis detector (left) and a refractive index (RI) detector (right). Unknown component is marked with a star.



Supplementary Figure 2. HPLC chromatogram of the concentration effect using Spin columns. The phage lysate (blue line) is compared with the concentrated Rhizobiophages (grey line). The arrow indicates the unknown byproduct.

3 Discussion

In this thesis, three studies were represented in order to gain deeper insight into the role of viruses in oligotrophic deep-subsurface sediments. Therefore, we perused several strategies, including experiments on Northern-German tidal flats as a model habitat.

3.1 Viral production in marine sediments

Viruses outnumber prokaryotic cells in both, marine surface and subsurface sediments (Engelhardt *et al*., 2014). By lysing prokaryotes, viruses are factors for prokaryotic mortality and thereby influencing microbial community-structures (Proctor & Fuhrman, 1990;Steward *et al*., 1996).

3.1.1 Viral production and viral abundance in subsurface sediments of tidal flats

The impact of viruses on the benthic microbial communities of surface sediments has been investigated by measurements of viral production, worldwide (Danovaro *et al*., 2008b). However, studies on subsurface sediments one meter below seafloor and their influence on deep-biosphere sediments are scarce. We have demonstrated that viral production rates in an anoxic subsurface sediments of the tidal-flat Site Janssand are in the range of surface sediments from other tidal flats and deep-sea sediments or even higher (Hewson & Fuhrman, 2003; Danovaro *et al*., 2008b; Corinaldesi *et al*., 2010). Since viruses depend on their hosts metabolism, their abundance and activity is directly linked to that of the respective prokaryotes (Middelboe *et al*., 2003; Danovaro *et al*., 2008b; Siem-Jørgensen *et al*., 2008). As reported in a recent study, high cell numbers at Site Janssand were based on the high input of fresh organic matter (Beck *et al*., 2008; Gittel *et al*., 2008; Beck *et al*., 2009) which supports the high viral numbers and production rates. Furthermore, we determined the virus-induced prokaryotic mortality ranging between 6 and 115% of the prokaryotic standing stock. This is in accordance with previous studies in sediments, in which virus-induced prokaryotic mortality ranged between 2 and 336 % of the prokaryotic standing stock per day (Hewson & Fuhrman, 2003; Glud & Middelboe, 2004; Siem-Jørgensen *et al*., 2008) or between 12 and 238 % of prokaryotic net production (Mei & Danovaro, 2004; Middelboe *et al*., 2006; Danovaro *et al*., 2008b). Accordingly, viruses need to be included in the analysis of the respective ecosystems.

3.1.2 Comparison of two methods for viral production rate measurements

In our study on anoxic subsurface sediments of Site Janssand we found out that the dilution method was much more sensitive than the incorporation of labeled ^{3}H-thymidine into viruses. One reason for this might be the different incubation times of both approaches that were needed to detect changes in viral numbers. For the dilution method, short incubation times of 6 hours were sufficient, whereas a minimum of 4 days and sometimes even stimulation with glucose were necessary for the radiolabeling method. Additionally, the latter method requires the application of a factor for the conversion of the measured radioactivity into viral production rates. It has been shown that conversion factors strongly depend on the respective environmental conditions and experimental procedure and therefore cannot be transferred from one environment to the other (Fuhrman & Azam, 1982; Steward *et al*., 1992; Teira *et al*., 2015). The establishment of an own conversion factor for anoxic subsurface sediments turned out to be highly challenging due to several issues discussed within the paper. Among them, we identified incomplete labeling of viral DNA as one factor. Furthermore, not all bacteria (e.g. methanogens and/ or sulfate reducers) might be able to incorporate the label (Michel & Bloem, 1993; Wellsbury *et al*., 1993; 1994). As for the dilution method, possible overestimation due to the procedure itself was also discussed in a comparison to other methods for viral production rate measurements. We concluded that viral production rates published in the literature are highly dependent on the chosen method and thus, need to be further evaluated in order to find the most accurate protocol.

3.1.3 Transferability of the methods to SPG sediments?

The comparison of the two methods for viral production during the pre-experiments with Janssand sediments revealed some critical points for the radiolabeling method, e.g. incomplete labeling and inability of incorporation by indigenous prokaryotes. The latter might not be an issue for the SPG as the entire sediment column is mostly oxygenated (Fischer *et al*., 2009; D'Hondt *et al*., 2015). However, the extremely low cell numbers and low respiration rates of the microbial communities within the SPG sediments would dramatically elongate the expected times that were already

necessary for highly active tidal-flat sediments. Moreover, the small cell sizes probably hamper the separation of prokaryotes and viruses by filtration.

As for the dilution method, measurements with SPG sediments might not be suitable. The principle behind the method is to assume that rates of viral production and decay in a habitat are in a steady-state. By diluting the samples, the virus background and the amount of virus-host encounters are reduced and thus, viral production is based on infections prior to the dilution (Wilhelm *et al*., 2002). Given the assumption that the majority of viruses in oligotrophic sediments such as with the SPG are likely temperate phages (Fuhrman, 1999; Weinbauer, 2004) together with the slow metabolism of the indigenous hosts, fast detectable increase in viral counts would rather be unlikely. Pre-experiments with sediments of Site 1371 (from the edge of the gyre), which is comparatively higher in activity than the sites in closer distance to the center, did not show any significant changes within the first hours of incubation. Additionally, the increasing virus-to-cell ratios detected in the SPG were interpreted as a potentially better preservation of viruses with depth (Engelhardt *et al*., 2014). Therefore, one of the main preconditions for the method might not be fulfilled.

3.2 Viruses as potential food-source for microbial communities in SPG sediments

As virus particles were shown to display a considerable amount of organic biomass in deeper layers of South Pacific Gyre sediments (Engelhardt *et al*., 2014), experiments were performed in order to test their putative utilization by indigenous microbial communities.

3.2.1 Active production of exoenzymes in microbial communities of SPG sediments

We confirmed that microbial communities of several SPG sediments are able to exhibit exoenzyme activities. Increasing phosphatase activities inversely correlated to the decreasing phosphate concentration with sediment depth. Our results are in accordance to findings of another study on deep-subsurface sediments of the Juan de Fuca Ridge (Engelen *et al*., 2008). However, this was still surprising as the measured phosphate concentration did not indicate phosphate limitation for the prokaryotes and the production of exoenzymes is relatively cost-intensive. We discussed the

possibilities of preservation or active production and concluded that preservation over such long time-scales is rather unlikely (Coolen & Overmann, 2000).

Especially by detecting aminopeptidase activities, the precondition for potential utilization of virus particles was fulfilled. The decreasing aminopeptidase activity with sediment depth was interpreted as diminished microbial activity. Additionally, decreasing activities with depth would also explain the increasing virus-to-cell ratio.

Calculated cell-specific activities based on published cell counts from D'Hondt and colleagues (2015) were extremely high compared to the literature e.g. (Boetius, 1995; Boetius *et al*., 1996; Coolen & Overmann, 2000) and might be biased due to difficulties in counting extremely low cell numbers (D'Hondt *et al*., 2015). Besides the investigations on bulk sediments, also starving cultures of isolates from SPG-sediment enrichments were shown to exhibit exoenzyme activities. Thus, it can be assumed that at least some bacteria in these sediments might be able to feed on phages.

3.2.2 Demonstration of phages as a food source by feeding experiments

Our hypothesis of putative utilization of virus particle by indigenous microorganisms within SPG sediments was further investigated by feeding experiments. Therefore, SPG isolates were incubated with phage concentrates of *Rhizobium radiobacter* strain P007 as sole carbon source. Two independent experiments revealed decreasing viral numbers over time compared to relatively constant numbers in cell-free and phage-free control cultures. These trends in viral counts were determined by two different methods. Thus, the results further supported the initial hypothesis. Parallel counting of bacterial numbers showed a subsequent increase in bacterial biomass. Unfortunately, HPLC analyses of the phage concentrates revealed the presence of glycerol as a potential additional food source, which was transferred into the cultures by the concentration procedure using spin-columns. Consequently, bacterial growth was likely the result of feeding on both, glycerol and phage particles. The same argumentation is also true for the experiments on SPG sediment that was amended with phage concentrates. We concluded that the viruses in the marine deep biosphere of the SPG might not only be the predators for indigenous microorganisms, but instead serve as a carbon source as an adaptation of the bacteria to the low availability of organic matter. However, the final evidence needs further experiments for example

with isotopically labeled phage particles. Furthermore, pre-washing of the spin column should reduce or remove the glycerol content.

3.2.3 Method-improvements for analyzing virus-host interactions

Pre-experiments with ^{13}C-labeled phages from *R. radiobacter* strain P007 were already performed. HPLC analysis of the phage concentrates revealed a second unknown peak in addition to the glycerol carried over by using the spin column. This substance was only detected in the phage concentrate that derived from the induction experiments of *R. radiobacter* incubated with ^{13}C-labeled glucose. Consequently, this unidentified substance might also be labeled and thus, could again prevent to follow the incorporation of ^{13}-labeled phage particles by the isolates or microbial communities from the SPG sediments. Hence, further purification of the phage concentrates by e.g. gradient centrifugation or testing of labeled glucose from other companies should be performed.

As an alternative method, labeling of either specific DNA-regions (e.g antirepressor gene) or parts of the protein capsules of Rhizobiophages with specific primers could be performed. The labeled component should be combined with a fluorogenic substance. Incorporation of the labeled fraction into DAPI-stained cells could then be followed by fluorescence microscopy. This method is known as phageFISH and has been established for studies on infection dynamics in a marine podovirus-gammaproteobacterial host model system (Allers *et al*., 2013). However, for the detection of bacteria feeding on viruses, this method also harbors some challenges. For example, cells would need to be able to incorporate the intact molecules, including the probe. As shown in this thesis, microbial communities and isolates of SPG sediments actively produce exoenzymes that could cleave the viral components prior to incorporation. There is a risk that fragmentation would result in the loss of the probe. However, this would have to be investigated.

3.3 Future perspectives

Although the studies presented in this thesis provide interesting new insights into virus-host interactions, there are much more questions to be answered regarding SPG sediments. In the following chapter, some ideas and requirements of new methods for further studies on SPG sediments will be presented.

3.3.1 Studying viral diversity in SPG sediments by using molecular methods

Due to the low primary production within the water column of the SPG (Jahnke, 1996), only low amounts of organic matter reaches the seafloor. However, differences occur from the edge of the gyre into the center. This is reflected by sediment thickness between 130 m at the edge (Site U 1371) and a minimum of ~15 m in the center of the gyre (Site U1368) (D'Hondt *et al*., 2015). The basement ages of the sediments differ between ~13.5 and 100 Myr (D´Hondt *et al*., 2011). Consequently, sediment age increases dramatically even in a centimeter scale. As virus-like particles were counted at all sites an even down to the basaltic crust, it might be interesting to investigate, whether also the viral communities show steep differences in composition with sediment depth as well as along the trophic gradient of the gyre.

In order to test this hypothesis, fingerprinting methods such as randomly amplified polymorphic DNA-PCR (short RAPD-PCR) on purified viruses extracts would be a possible tool. This PCR is based on short primers that unspecifically (randomly) target DNA fragments within the template. The resulting patterns of different sites and depth layers could be compared to each other. The application of a more specific primer for the whole viral community is not possible, as viruses do not harbor an overall conserved region for all species within their DNA (Paul *et al*., 2002; Sullivan *et al*., 2008). Alternatively, it would be possible to choose a representative family within the viruses that have some uniform genes (Rohwer & Edwards, 2002). Until now, studies have mainly focused on investigating viral members of the *Caudovirales* (e.g. *Myoviridae*) that account for around 96% of the viruses identified so far (Filee *et al*., 2005; Ackermann, 2007).

Another method for studying viral diversity is the metagenomic analysis of either purified phage-extracts or even the combined prokaryotic and viral community of different sites and sediments depths. The latter would open the window not only to

study the viruses, but to directly compare them to their hosts. Analyzing the prokaryotic hosts would additionally enable the characterization and relative estimation of the temperate-phage fraction within the sediments. The decisive advantage of metagenomic analyses is that there is no need for a single conserved region within all viruses (Martínez *et al*., 2014).

3.3.2 Challenges of molecular analyses

Investigating viral diversity requires the preparation of pure viral extracts, including the degradation of external prokaryotic DNA. Several protocols have been published for this task (Danovaro *et al*., 2001; Steward, 2001; Chen *et al*., 2006). Usually, they include dislodgement and separation (filtration) of the phages from the sediment particles (Danovaro *et al*., 2001), followed by a concentration step and a DNase treatment in order to exclude external prokaryotic DNA (Engelhardt *et al*., 2013). The low viral biomass requires an additional step before starting molecular analyses as the methods need a certain amount of virus material. Zablocki *et al*. (2014) published a study on antarctic soils, where they faced similar problems of low viral DNA content after the extraction and concentration procedure, although they were able to collect kilograms of surface soils. Hence, they applied whole genome amplification (WGA) by using a Phi29 polymerase on their samples. This polymerase has been proven to be highly efficient in amplifying circular and linear DNA in one single step (Blanco *et al*., 1989; Johne *et al*., 2009) and was first applied on viruses by Rector *et al*. (2004). Consequently, this method might be a helpful tool for the relatively low viral biomass within the SPG sediments.

3.3.3 Analysis of the lysogenic fraction of SPG microbial communities

Despite the benefits of molecular analyses, applying cultivation-based studies opens the possibility to investigate physiological features as well as following virus-host relations in more detail. For example, it would be interesting to determine the lysogenic fraction of SPG microbial communities by induction experiments with sediment slurries and compare it to the remaining fraction. Therefore, counting of prokaryotic cells would need to be adjusted to deep subsurface sediments after the

protocol of Kallmeyer *et al*. (2008). Additionally, a sensitive counting method for viruses in sediments should be developed.

Moreover, by extending the experiment over longer times, it could be tested whether the surviving microbial community would be able to take advantage on the released fresh organic matter of the lysed cells. Therefore, performing analysis on changing organic matter content would be recommended.

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Thanks

Zuallererst möchte ich meinen Eltern Christine und René, meinem langjährigen Lebensgefährten Martin und meiner Familie von ganzem Herzen danken. Eure uneingeschränkte und bedingungslose Unterstützung in allen Lebenslagen hat mich an diesen Punkt im Leben gebracht.

Mein besonderer Dank gilt meinen Betreuern Heribert Cypionka und Bert Engelen für die Anleitung und die konstruktiven Gespräche während der letzten Jahre sowie die Möglichkeit, mich in dieser Arbeitsgruppe einzubringen und weiterzuentwickeln. Außerdem möchte ich mich ganz herzlich für eure Motivationsarbeit und die Aufmunterungen bedanken!

An die Mitglieder unserer Mädelsrunde – Saranya, Marion und Sarah – ohne euch wäre es nicht halb so lustig gewesen, sowohl im Labor als auch außerhalb davon!!! Unvergessen bleiben die zahllosen Koch- und Backaktionen, Unternehmungen und die gemeinsame Zeit in Indien. Danke für alles. Ich bin glücklich und fühle mich geehrt, euch als meine Freunde zu haben!

Ich bedanke mich bei allen aktuellen und ehemaligen Mitgliedern der Arbeitsgruppe Paläomikrobiologie. Jana und Frank, danke für den Spaß und die Hilfe im Labor und die Knuddeleinheiten☺; Verona für die geduldige Hilfe bei der langwierigen, aber schlussendlich erfolgreichen Schreibarbeit an der ersten Studie; Maya, Tim, Oscar und Christian für die Abwechslung und den Spaß im Laboralltag. Ganz besonders werden mir die Weihnachtsfeiern, inklusive kreativer Vorbereitung in Erinnerung bleiben.

Vielen Dank auch an alle anderen, die zu der Vollendung dieser Arbeit beigetragen haben, besonderes an meine Master-Studentinnen Lisa und Sarah, für das Durchhaltevermögen, auch wenn mal nicht alles gleich funktionierte.

Last but not least, bedanke ich mich bei den Mitgliedern der Arbeitsgruppe Simon für die kollegiale Zusammenarbeit sowie die vielen gemeinsamen Unternehmungen, die immer wieder Schwung in den Alltag gebracht haben. Besonders zu erwähnen sind dabei Sara (ich hatte unheimlich viel Spaß mit unseren Foto-Ausflügen) und Insa (mir werden unsere gemeinsamen Anime-Abende fehlen).

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Zusätzlich erkläre ich, dass diese Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat.

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