HELDA
HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

UNIVERSITY OF HELSINKI

https://helda.helsinki.fi

# The first known virus isolates from Antarctic sea ice have complex infection patterns

Luhtanen, Anne-Mari

2018-04

Luhtanen , A-M , Eronen-Rasimus , E L , Oksanen , H M , Tison , J-L , Delille , B , Dieckmann , G S , Rintala , J-M & Bamford , D H 2018 , ' The first known virus isolates from Antarctic sea ice have complex infection patterns ' , FEMS Microbiology Ecology , vol. 94 , no. 4 , 028 . https://doi.org/10.1093/femsec/fiy028

http://hdl.handle.net/10138/300595
https://doi.org/10.1093/femsec/fiy028

acceptedVersion

*Downloaded from Helda, University of Helsinki institutional repository.*

*This is an electronic reprint of the original article.*

*This reprint may differ from the original in pagination and typographic detail.*

*Please cite the original version.*

# Host specificity and temperature adaptation of the first known Antarctic sea-ice virus isolates

Anne-Mari Luhtanen[1,2,3], Eeva Eronen-Rasimus[1], Hanna M. Oksanen[2], Jean-Louis Tison[4], Bruno Delille[5], Gerhard S. Dieckmann[6], Janne-Markus Rintala[3,7] and Dennis H. Bamford[2]

[1]Marine Research Centre, Finnish Environment Institute, Helsinki, Finland, [2]Programme on molecules, cells and systems, University of Helsinki, Helsinki, Finland, [3]Tvärminne Zoological Station, University of Helsinki, Hanko, Finland, [4]Laboratoire de Glaciologie, DGES, Université Libre de Bruxelles, Belgium, [5]Unité d'Océanographie Chimique, Université de Liège, Belgium, [6]Alfred Wegener Institute Helmholtz Center for Polar and Marine Research, Bremerhaven, Germany, [7]Department of Environmental Sciences, University of Helsinki, Helsinki, Finland

Correspondence:
D.H. Bamford, Programme on molecules, cells and systems, FIN-00014 University of Helsinki, Finland. E-mail: dennis.bamford@helsinki.fi

## Conflict of Interest

The authors declare no conflict of interest.

For Peer Review

**Abstract**

Viruses are recognized as important actors in ocean ecology and biogeochemical cycles, but many details are not yet understood. We participated in a winter expedition to the Weddell Sea, Antarctica, to isolate viruses and to measure virus-like particle abundance (flow cytometry) in sea ice. We isolated 59 bacterial strains and the first four Antarctic sea-ice viruses known (PANV1, PANV2, OANV1, and OANV2), which grow in bacterial hosts belonging to the typical sea-ice genera *Paraglaciecola* and *Octadecabacter*. The viruses were cold-active and specific for bacteria at the strain level, although OANV1 was able to infect strains from two different classes. Both PANV1 and PANV2 infected 11/15 isolated *Paraglaciecola* strains that had almost identical 16S rRNA gene sequences, but the plating efficiencies differed among the strains, whereas OANV1 infected 3/7 *Octadecabacter* and 1/15 *Paraglaciecola* strains and OANV2 1/7 *Octadecabacter* strains. The results showed that virus-host interactions can be very complex and that the viral community can also be dynamic in the winter-sea ice.

For Peer Review



ScholarOne Support 1-434/964-4100

## Introduction

Almost 10% of the world´s ocean is covered by sea ice at least once per year, which makes it one of the largest biomes on Earth (Dieckmann and Hellmer 2010). Although being a cold and harsh environment, sea ice is full of life. Specialized organisms live inside brine channels and pockets that are formed during freezing conditions, when salts and nutrients from the seawater become concentrated between the ice crystals (Thomas and Dieckmann 2002). The sea-ice microbial community consists of protists, bacteria, archaea, and their viruses (Maranger *et al.* 1994; Mock and Thomas 2005; Arrigo *et al*. 2010; Deming and Collins 2017). Here, we use the term bacteria instead of prokaryotes, even if in some cases archaea may also be involved. Microbes affect the biogeochemical properties of the sea ice, gas exchange between the ocean and atmosphere, and provide food for ice-associated animals, e.g. krill (Arrigo and Thomas 2004).

Viruses are the most numerous of life forms and are presumed to play important roles in the biogeochemical cycles of the oceans (Fuhrman 1999; Suttle 2007). The most commonly found viruses, bacteriophages (phages) i.e. viruses infecting bacteria, are possibly the main cause of bacterial mortality (Weinbauer 2004; Suttle 2007). Since viruses can multiply only within their host cells, their activity is dependent on the abundance and activity of their hosts (Maranger *et al*. 1994; Marchant *et al*. 2000). Due to their host specificity, they are crucial to control of bacterial community composition and activity (Proctor and Fuhrman 1990; Wommack and Colwell 2000; Suttle 2005; 2007). However, most studies of marine environments are conducted in the water column, whereas knowledge of viruses and their functions in sea ice is still very limited.

The numbers of virus-like particles (VLPs) measured, range between $10^5$ and $10^8$ $ml^{-1}$ in bulk Arctic and Antarctic sea ice from spring to autumn. The lowest values


ScholarOne Support 1-434/964-4100
For Peer Review

have been observed during the winter in Antarctic bulk ice (Paterson and Laybourn-Parry 2012), whereas the highest numbers of VLPs occur during freezing or spring algal mass growth (Maranger *et al*. 1994; Collins and Deming 2011). However, VLPs may also contain particles other than viruses, e.g. gene transfer agents or membrane vesicles (Forterre *et al*. 2013; Soler *et al*. 2015). VLPs are positively correlated with bacterial abundance, activity, and chlorophyll-*a* (chl-*a*) concentrations (Maranger *et al*. 1994; Gowing *et al*. 2002, 2004). In aquatic environments, a typical virus-to-bacteria ratio (VBR; more precisely VLP-to-prokaryotic cell ratio) is 10:1 (Maranger and Bird 1995). Bacterial and viral density specifies their contact rate, which is one of the key controls in virus-host interactions. The semienclosed environment of brine channels may increase this contact rate, especially during winter, when the brine channels are narrower and the brine even more concentrated (Wells and Deming 2006a). Sea ice may, therefore, be a place where virus-host interactions can be enhanced, compared with the open ocean. To understand these effects, virus-host systems need to be isolated to examine their interactions in detail.

To the best of our knowledge, only three virus-host systems to date have been isolated from Arctic and seven from Baltic sea ice (Borriss *et al*. 2003; Luhtanen *et al*. 2014, Yu *et al*. 2015), but none from Antarctic sea ice. The viruses isolated represented different phage morphologies. The Arctic virus isolates *Shewanella* phage 1a and *Colwellia* phage 21c are icosahedral viruses with either a contractile or noncontractile tail, resembling double-stranded DNA (dsDNA) phages of the order *Caudovirales* (Borriss *et al*. 2003; 2007). The filamentous nonlytic phage f327 from the Arctic infects a *Pseudoalteromonas* strain and is reminiscent of viruses in the family *Inoviridae* (Yu *et al*. 2015). In addition, seven tailed icosahedral dsDNA phages infecting strains from either *Flavobacterium* or *Shewanella* were isolated from Baltic Sea ice (Luhtanen *et al*. 2014; Senčilo *et al*. 2015). All virulent sea-ice phage isolates have a narrow host range, are cold-active

For Peer Review


ScholarOne Support 1-434/964-4100

(capable of infection and production at ≤ 4 °C; Wells and Deming 2006b), and produce plaques (clear zones on bacterial lawns used to determine the number of infectious viruses) only at the lower end of their host bacterial temperature growth range (Borriss *et al.* 2003; Luhtanen *et al*. 2014). In addition, a cold-active siphophage 9A was isolated from Arctic nepheloid layer seawater (Wells and Deming 2006b) for *Colwellia psychrerythraea* strain 34H, isolated originally from Arctic shelf sediments (Huston *et al*. 2000). It was reminiscent of the isolates from sea ice, because it also has a narrow host range, is cold-active, and has a more restricted growth temperature range than the host bacteria.

Here, we report the isolation of the first cultivable phage-host systems and VLP abundance from the winter-sea ice in the Weddell Sea, Antarctica. Studying phage-host systems gives us valuable information on sea-ice microbial communities and their potential roles in the sea-ice ecosystem.

For Peer Review

## Materials and Methods

### Sea-ice sampling and ice properties

Pack ice samples were collected from the Weddell Sea (Antarctica) during the austral winter as part of the Antarctic Winter Ecosystem Climate Study (AWECS) aboard R/V Polarstern in June–August 2013 (leg ANT-XXIX/6). Sampling was performed either with a metal basket (pancake ice at stations 486, 488, and 489) or using a motorized, trace-metal-clean (electropolished steel) CRREL-type ice-coring auger (Lannuzel *et al*. 2006), 14 cm in diameter. For this study, full-depth ice cores (one or two) were taken from 10 locations (ice-stations 486, 488, 489, 493, 496, 500, 503, 506, 515, and 517) (Table 1), as described in Tison *et al*. 2017. Bulk ice was used, because some of the microbes may have been attached to the brine channel walls and could have been lost if only the liquid brine were sampled (Meiners *et al*. 2008). The ice cores were cut into one, three, five, or seven layers, depending on the ice thickness, and crushed gently with a hammer inside a polyethylene plastic bag. The corresponding layers of the possible sibling ice cores were pooled (Table 1). The VLP abundances were measured from these layers. The bacterial abundances (Table 1), bacterial production (measured as thymidine incorporation), and bacterial community composition analyses from these ice cores have been published elsewhere (Eronen-Rasimus *et al*. 2017). For the isolation work, we used the ice samples from first-year ice-station 500 and early second-year ice-station 515a (Tison *et al*. 2017). The surface parts of the 515a core were removed to minimize contamination, and the core was cut into 12 layers (~14 cm each; Table 2), using an electric-band saw sterilized with 70% ethanol. The 12 layers were used separately to isolate the bacterial strains. The bacterial community composition of ice core 515a has also been published under the name 515 T (0–56 cm), M (56–126 cm), and B (126–166 cm) (Eronen-Rasimus *et al*. 2017). For virus isolation, bulk ice from station 500 and core 515a was used. The ice samples were left to melt in sterilized containers at 4 °C overnight, after which

For Peer Review


ScholarOne Support 1-434/964-4100

the remaining ice was melted in a water bath with continuous shaking (Rintala *et al*. 2014). After melting, the samples were immediately transferred back to 4 °C.

The pancake ice at stations 486 and 488 was 6 cm and 35 cm thick, the first-year ice at stations 489–506 and 517 was 37–90 cm, and the early second-year ice at station 515 was 179 cm (Table 1, Tison *et al*. 2017). The ice temperature varied between -1.7 and -12.6 °C, with a median temperature of -3.8 °C and ice chl-*a* median concentration of 2.4 µg $l^{-1}$(Tison *et al*. 2017). The ice at station 500 was dominated by frazil ice and at station 515 by mixed columnar and frazil ice (Tison *et al*. 2017). The brine salinity varied from 122 to 40 practical salinity units (Tison *et al*. 2017).

**Isolation of the bacterial strains**

Isolation of the bacterial strains was started immediately after the 12 layers of the ice core 515a samples were melted. The strains were isolated by plating 100 µl of the melted sample on (*I*) ZoBell plates (1000 ml Southern Ocean water, 5 g peptone, 1 g yeast extract, 15 g agar; Helmke and Weyland 1995; Middelboe *et al*. 2003), (*II*) concentrated ZoBell plates (the Southern Ocean water was concentrated by boiling to half of the initial volume, otherwise similar to ZoBell), and (*III*) Modified Oxford (MOX) agar plates (750 ml seawater, 250 ml ultrapure water, 1 g $KNO_4$, 0.2 g yeast extract, 10 mg $FePO_4$, 2 g HEPES, 12 g agar). The plates were incubated at 4 °C and transported from R/V Polarstern to the home laboratory with temperature-controlled courier transportation at -2.6 °C to +6.1 °C (World Courier, AmerisourceBergen, Stamford, CT, USA). After 1 month of incubation in the dark at 4 °C, the various colony morphotypes were picked and colony-purified at three consecutive times. Colony purification and cultivation were done on modified ZoBell medium, Reef Crystal (RC) medium: 33 g Reef Crystals, Aquarium Systems Inc. Sarrebourg, France, 1000 ml ultrapure water, 5 g peptone, 1 g yeast extract). The agar concentration for the plates was 1.5% (w/v). The strains were

For Peer Review


ScholarOne Support 1-434/964-4100

grown aerobically in RC medium at 4 °C for 7 days and stored at -80 °C, supplemented with 15% (v/v) glycerol.

**Identification of the bacterial strains**

The colony-purified strains were identified by 16S rRNA gene sequencing. The genomic DNA was isolated with an UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). The partial 16S rRNA genes were amplified with PCR, using primers F27 (Sait *et al*. 2003) and R1406 (Lane *et al*. 1985) or pA and pGr (Edwards *et al*. 1989). The Sanger sequencing was performed at the DNA Sequencing and Genomics Unit, Institute of Biotechnology (University of Helsinki), using primers pDr, pE, and pFr (Edwards *et al*. 1989). The taxonomic identification of the strains was done with SILVA Incremental Aligner (SINA) Alignment Service (version 1.2.11, 10.6.2016, Pruesse *et al*. 2012). The partial 16S rRNA gene sequences of the isolated bacterial strains are deposited in the NCBI GenBank database under accession numbers KY194799–KY194857 (Table 2).

**Phylogenetic analysis of the 16S rRNA sequences**

For the phylogenetic tree, alignment was performed with SINA Aliqnment Service (version 1.2.11, minimum identity: 0.8, Pruesse *et al*. 2012). Reference sequences were selected, based on SINA sequence matching, and for the nontype-strain sequence matches, their type-strain representatives were also added (EZBioCloud Database (Yoon *et al*. 2017). The 16S rRNA sequence of *Sulfolobus tokodaii* (AB022438) was used as an outgroup in the alignment. After the alignment, all sequences were truncated according to the sequence length, and the bootstrapped (1000) maximum-likelihood tree was constructed, using RAxML (version 8.2.0; Stamatakis 2014), with the GTRGAMMA evolution model. The tree was visualized with the Interactive Tree Of Life (iTOL) online tool (Letunic and Bork 2007).

For Peer Review


ScholarOne Support 1-434/964-4100

**Abundance of virus-like particles by flow cytometry**

The abundances of the VLPs were analyzed with flow cytometry from all the ice layers originating from the 10 stations (Table 1). Sample handling and measurements were done according to Brussaard *et al*. (2010), except that paraformaldehyde [1% (v/v) in phosphate-buffered saline (PBS), Alfa Aesar GmbH & Co KG, Karlsruhe, Germany] was used as a fixative. The samples were stained with SYBR Green I (Sigma-Aldrich Inc., Saint Louis, MO, USA) at room temperature. To measure the background, virus-sized particles were removed from the control samples by ultrafiltration (Amicon Ultra-15 concentrators, MWCO 100 000 Da; Merck Millipore, Billerica, MA, USA; 4000 *g*, 4 min, 4 °C), and the controls were processed in the same way as the actual samples. Earlier isolated and purified 1/4, 1/32, 1/40, 1/41, 1/44, 3/49 phage particles (Luhtanen *et al.* 2014) were used as controls to define the virus population, and Fluoresbrite 0.5-µm microspheres (Polysciences Inc., Warrington, PA, USA) were used as a size standard. Enumeration of the diluted samples (dilution factor 10; molecular biology grade TE buffer, AppliChem GmbH, Darmstadt, Germany) was carried out, using a CyFlow Cube 8 (Partec GmbH, Münster, Germany) flow cytometer equipped with a 488-nm laser. The data were analyzed, using FCS Express 4 software (De Novo Software, Glendale, CA, USA), and the numbers were corrected for dilution. The VBR was defined, using the bacterial abundance results published earlier (Eronen-Rasimus *et al*. 2017).

**Isolation of viruses**

For virus isolations, the melted layers of the station 500 core were pooled, as were the layers of core 515a. The melted ice was filtered through a 0.22-µm Durapore Membrane polyvinylidene difluoride (PVDF) filter (EMD Millipore Corporation, Billerica, MA, USA) to remove cellular organisms. The filtrates were concentrated 50 times, using Amicon Ultra-15 concentrators (MWCO 100 000 Da) and centrifugation (2000 *g*, 5 min, 4 °C). The concentrates were stored in 15% (v/v)

For Peer Review


ScholarOne Support 1-434/964-4100

glycerol at -80 °C and transported in liquid nitrogen to the home laboratory. All isolated and colony-purified bacterial strains (Table 2, Figure 1) that were able to form a bacterial lawn were used to isolate viruses. Virus isolation and further cultivation were done with a plaque assay. For virus isolation, 10 µl and 100 µl of the concentrated sample with 200 µl of dense host bacterial suspension (grown for 7 days in RC medium at 4 °C) and 3 ml of melted RC top-layer agar [0.4% (w/v) agar; 43 °C] were mixed and poured on RC plates. After 1–3 weeks of incubation at 4 °C, the plaques were individually picked and plaque-purified three consecutive times with the plaque assay. For the plaque assay, 100 µl of suitable virus dilution, 200 µl of host liquid culture, and 3 ml of RC top-layer agar were mixed and poured on RC plates, which were incubated for 5–7 days at 4 °C.

**Production and purification of viruses**

The virus lysates were prepared with the plaque assay as described above, using semiconfluent plates. The top-layer agar was collected and mixed with 2 ml of RC broth per plate. The suspension was incubated for 1 h at 4 °C with shaking, after which the cell debris and agar were removed (centrifugation: 10 000 *g*, 30 min, 4 °C). Virus precipitation from the lysates was optimized with various ammonium sulfate concentrations [50%, 60%, 70%, and 80% (w/v)], using either saturated solution or ammonium sulfate powder (Boulanger and Puvion 1973; Burgess 2009). Ammonium sulfate was mixed with or dissolved in the lysate for 1 h at 4 °C with shaking. The precipitated viruses were collected (centrifugation: 14 000 *g*, 60 min), washed with SM buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$; Borriss *et al*. 2003) with or without 0.01% (w/v) gelatin and dissolved in SM buffer on ice. The virus aggregates were removed (centrifugation 9300 *g*, 10 min, 4 °C), and the viruses in the supernatant were subjected to rate-zonal centrifugation (153 208 *g*, 30–70 min, 10 °C), using 10–30% (w/v) linear sucrose gradients in SM buffer (Anderson *et al*. 1966; Lawrence and Steward 2010). The gradients were fractionated (12 fractions), and the infectivity (plaque assay),

For Peer Review


ScholarOne Support 1-434/964-4100

absorbance (260 nm), and the protein, nucleic acid, and lipid contents of the fractions were determined (see below). The virus particles in the light-scattering zones were collected with differential centrifugation (104 087 *g*, 3 h, 10 °C), and the particles were dissolved in SM buffer overnight on ice.

**Virus particle analyses**

The absorbance values (260 nm) of the 12 sucrose gradient fractions were measured with an Eppendorf BioPhotometer D30 (Eppendorf AG, Hamburg, Germany), using 30% (w/v) sucrose in SM buffer as a blank sample. The viral structural proteins were separated with SDS-PAGE (16% (w/v) acrylamide; Olkkonen and Bamford 1989). The SM buffer used in the protein analysis samples did not contain gelatin. The protein concentrations were determined with a Coomassie Blue-based method (Bradford 1976), using bovine serum albumin as a standard. For the SDS-PAGE, when appropriate, the samples were concentrated with 10% (v/v) trichloroacetic acid precipitation (30 min, on ice). The precipitate was collected (centrifugation: 16 200 *g*, 30 min, 4° C). The resolving gels were stained with Brilliant Blue R (Sigma-Aldrich) for proteins and, when appropriate, stained with Sudan Black B (Sigma-Aldrich) for lipids and the stacking gels with ethidium bromide for nucleic acids. For the lipid-staining control, purified PRD1 particles (Bamford and Bamford 1991) were used as a control. To test the sensitivity of the viruses to Triton X-100, viruses were incubated in 0.1% (v/v) and 0.01% Triton X-100 (in SM buffer) for 3 h and 24 h at 4 °C. SM buffer was used as a control. The infectivity of the viruses tested was determined with a plaque assay. The sensitivity of the host organisms to Triton X-100 was analyzed similarly, except that the number of colony-forming units was determined by plating. For TEM, the purified virus particles were negatively stained for 20 s, using 2% (w/v) uranyl acetate (pH 7), 3% (w/v) uranyl acetate (pH 4.5), or 1% potassium phosphotungstate (pH 7). A JEOL JEM-1400 TEM (Electron

For Peer Review

Microscopy Unit, Institute of Biotechnology, University of Helsinki) was used with 80-kV tension for detailed investigation of the viruses.

**Temperature ranges of host growth and virus infection**

The growth of the original three host strains at different temperatures was determined by plating 100 µl of diluted bacterial suspension on RC plates, which were incubated at 0, 4, 10, 15, or 20 °C for 60 days. The infection ability of the bacteriophages at different temperatures was tested with a plaque assay. The host suspensions used for the plaque assay were incubated at 4 °C. The plates were incubated at 0, 4, 10, or 15 °C for 20 days.

**Virus-prokaryote interactions**

All the bacterial isolates that were able to grow as a lawn on a plate were tested for their sensitivity to the isolated viruses (Table 2, Figure 1). Ten microliters of undiluted and 100-times diluted virus lysates were spotted on the host strain lawns. RC medium was used as a negative control and the original virus-host pair as a positive control. The plates were incubated at 4 °C for 7–14 days. All positive results were verified with a plaque assay, using suitable dilutions of the virus. The EOP was calculated according to the plaque count obtained with the target strain, compared with that obtained with the isolation host strain.

For Peer Review


ScholarOne Support 1-434/964-4100

## Results

### Sea-ice bacterial isolates

We isolated 59 bacterial strains from core 515a (Table 2). The majority of the isolates (~59%) were classified as members of the genera *Glaciecola* or *Paraglaciecola* (Figure 1). Only one strain, IceBac 363, was isolated from the coldest top layer and belonged to the genus *Halomonas*. The remaining strains were identified as members of the genera *Octadecabacter* (seven strains), *Polaribacter* (seven strains), *Marinobacter* (three strains), *Pseudoalteromonas* (three strains), *Colwellia* (two strains), or *Paracoccus* (one strain). All isolated bacterial strains belonging to the same genus had identical or nearly identical (identity 99.3–100%) partial 16S rRNA gene sequences (at minimum 1290 base pairs, bp).

### Virus-like particle abundance and virus-to-bacteria ratios in sea ice

The mean VLP abundance was $1.1\times10^{6}$ $ml^{-1}$ (range $1.9\times10^{5}$–$4.9\times10^{6}$ $ml^{-1}$) in the bulk ice, with the highest numbers at stations 500 and 515 ($0.7\times10^{6}$–$4.9\times10^{6}$ $ml^{-1}$; Table 1). The mean VBR was 5.3 (range 0.7–13.4; Table 1). The highest VBR values were at station 503 (9.5–13.4; Table 1) and the lowest at station 515 (0.7–2.9; Table 1).

### Sea-ice bacteriophage isolates

Forty-eight out of the 59 bacterial strains were able to form a bacterial lawn on a plate and were consequently used to screen phages (Table 2). Four phages were obtained, cultivated, and purified in the laboratory (Table 3). The phages were named after the isolation host genus, area of isolation, and the initials of notable persons in this study (Krupovic *et al*. 2016). The names and their abbreviations are *Paraglaciecola* Antarctic GD virus 1 (PANV1), *Paraglaciecola* Antarctic JLT virus 2 (PANV2), *Octadecabacter* Antarctic BD virus 1 (OANV1), and *Octadecabacter* Antarctic DB virus 2 (OANV2). Phages PANV1 and PANV2

For Peer Review



ScholarOne Support 1-434/964-4100

originated from station 500 and were isolated for the same host (IceBac 372), which was classified as *Paraglaciecola psychrophila* (similarity 99.9%). PANV1 produced clear plaques 3–4 mm in diameter, whereas the PANV2 plaques of 3–5 mm in diameter had a clear center surrounded by a turbid halo. Phages OANV1 and OANV2 were isolated from core 515a for two different bacterial hosts, IceBac 419 and IceBac 430, respectively, both identified as *Octadecabacter antarcticus*. Both phages produced clear plaques, but the diameters were different (3–6 mm for OANV1, 6–8 mm for OANV2). The optimized phage lysate titers varied from ~$6\times10^{9}$ to ~$5\times10^{11}$ plaque-forming units (pfu) $ml^{-1}$, depending on the phage (Table 3). The infectivity of the lysates was retained for several months when stored at 4 °C. All three host strains originated from different layers in the ice core (Table 2).

**Purification and characterization of the phages**

To characterize the phages, virus purification methods were optimized, based on ammonium sulfate precipitation and rate-zonal ultracentrifugation, following the recovery and purity of the infectious viruses at each step. Using ammonium sulfate precipitation, 25–54% of the infectious viruses were recovered, depending on the virus (Table 4). Both the ammonium sulfate powder and the saturated solution resulted in similar yields. PANV1 and PANV2 were precipitated with 50% ammonium sulfate, whereas OANV1 and OANV2 needed 80%. The precipitated particles were further purified with rate-zonal ultracentrifugation, and significant amounts of various noninfectious protein impurity species were detected at the top of the sucrose gradient. For all viruses, a single visible infectious light-scattering zone was detected in the middle of the sucrose gradient (Figure 2). This zone contained several proteins of different sizes that were unique for each virus. PANV1 and OANV2 had one major protein type in sizes of ~55 and ~35 kDa, respectively, while PANV2 and OANV1 had two major protein types (~40 and ~12 kDa in PANV2; ~35 and ~15 kDa in OANV1). A peak in the absorbance was detected in the same light-scattering zone, as were the nucleic acids when visible.

For Peer Review


ScholarOne Support 1-434/964-4100

Lipids could not be detected from the gels after Sudan Black staining (not shown). In addition, treatment of phages with the nonionic detergent Triton X-100 did not affect their infectivity, suggesting that the virus particles did not contain a lipid component. Specific infectivities (~2 –9×$10^{12}$ pfu $mg^{-1}$ protein) calculated for the purified phages showed that all virus samples were highly infectious after biochemical purification (Table 4). After the final concentration step with differential centrifugation, the recoveries of infectious viruses varied from ~10% to 20% (Table 4).

Transmission electron microscopy (TEM) of the purified particles (Figure 3) showed that phage PANV1 had a rigid, contractile tail typical of myoviruses. Its average tail length was ~58 nm and head diameter ~71 nm. PANV2 infecting the same host had an ~89-nm noncontractile tail characteristic of the siphoviruses and an ~52-nm head diameter (Table 3). OANV1 also had a typical siphovirus tail, with an average length of ~83 nm and a head ~50 nm in diameter, whereas OANV2 seemed to have a very short tail typical of podoviruses and a head ~53 nm in diameter (Table 3).

**Temperature range tests for host growth and phage infection**

All the bacterial host strains (IceBac 372, IceBac 419, and IceBac 430) were able to form colonies at the temperatures from 0 °C to 15 °C, but not at 20 °C (Table 5), and were therefore classified as psychrophiles (Morita 1975). The effect of temperature on phage infection (plaque formation) was tested at temperatures supporting the growth of the hosts. All the phages were able to infect their original host only at 0 °C and 4 °C, but not at higher temperatures (Table 5). PANV1 and PANV2 produced plaques at 0 °C and 4 °C in 6 days, but OANV1 and OANV2 produced plaques at 0 °C in 14 days and at 4 °C in 6 days.

**Phage-bacteria interactions**

For Peer Review


ScholarOne Support 1-434/964-4100

The sensitivity of all the 48 isolated bacterial strains (that were able to grow as a bacterial lawn) to the isolated phages was tested (Table 2; Figure 1). In all, 17 strains (13 *Paraglaciecola* strains and 4 *Octadecabacter* strains) were sensitive to at least one of the phages (Figure 1). Of 16 *Paraglaciecola* isolates, IceBac 372 was sensitive to three phages: PANV1, PANV2, and OANV1. Ten other *Paraglaciecola* strains were sensitive to both PANV1 and PANV2, but with different plating efficiencies (EOPs), two strains were sensitive to either PANV1 or PANV2, but with low EOP, and three strains could not be infected. All seven *Octadecabacter* strains had 100% identical 16S rRNA gene sequences (within 1289 bp, Figure 1). However, only four out of seven *Octadecabacter* strains were sensitive to either OANV1 or OANV2 and showed different EOPs.

Both the PANV1 and PANV2 phages were able to infect 12 different *Paraglaciecola* strains with different EOPs. However, each of them infected only a certain strain (IceBac 417 or IceBac 420, respectively) that the other could not (Figure 1). In addition to its original host strain (IceBac 419, *Octadecabacter*), OANV1 was able to infect two other *Octadecabacter* strains, but with lower EOP. It also produced plaques with high EOP in the strain IceBac 372 (*Paraglaciecola*), which was the isolation host for PANV1 and PANV2 (Figure 1). Consequently, OANV1 was able to infect strains representing two classes: Gammaproteobacteria (*Paraglaciecola*) and Alphaproteobacteria (*Octadecabacter*). In contrast, phage OANV2 was able to infect only IceBac 430 (*Octadecabacter*; Figure 1).

For Peer Review


ScholarOne Support 1-434/964-4100

## Discussion

We isolated and purified four Antarctic sea-ice phages (PANV1, PANV2, OANV1, and OANV2) that could be maintained and cultivated under laboratory conditions. They were cold-active (capable of infection and production at ≤ 4 °C; Wells and Deming 2006b), infecting bacterial strains belonging to the typical sea-ice bacterial genera *Paraglaciecola* or *Octadecabacter*. The viruses were specific for host recognition at the strain level, even though OANV1 was able to infect bacterial strains from two different classes. The highest VLP abundances were in the samples where bacteria were most abundant and active (Eronen-Rasimus *et al*. 2017).

### Isolation of sea-ice bacteria and phages

We isolated 59 bacterial strains belonging to nine different genera from Antarctic winter-sea ice. The ice was melted with the direct-melting method, which has been shown to result in viable bacteria counts similar to those obtained by melting with seawater addition (Helmke and Weyland 1995), even if it may cause osmotic stress due to the rapid salinity changes. The isolated strains belonged to the following genera: *Colwellia, Glaciecola, Halomonas, Marinobacter, Octadecabacter, Paracoccus, Paraglaciecola* (formerly *Glaciecola*; Shivaiji and Reddy 2014)*, Polaribacter,* and *Pseudoalteromonas*, all of which are common members in the sea-ice bacterial community (Bowman *et al*. 1997; Brinkmeyer *et al*. 2003; Deming and Collins 2017). The majority of these genera were also abundant in isolation ice core 515a, based on bacterial community composition analysis (see results in Eronen-Rasimus *et al*. 2017).

In addition, four unique phages were isolated from the sea ice with the direct-melting and plaque assay methods, even though the viruses were exposed to a 43 °C temperature for a short time during the plaque assay. This has also been

For Peer Review



ScholarOne Support 1-434/964-4100

successfully used previously (Borriss *et al.* 2003), and at least some of the cold-adapted phages can apparently tolerate high temperatures for a short time. Wide temperature tolerance can be beneficial to phage survival in natural environments throughout the various seasons.

Presumably, virus reproduction is most effective when the number of susceptible hosts is high and active (Thingstad and Lignell 1997). The hosts of the bacteriophages OANV1 and OANV2 belonged to the genus *Octadecabacter*, which was abundant in phage isolation core 515a (Eronen-Rasimus *et al*. 2017). The genus *Paraglaciecola* (host of phages PANV1 and PANV2) could not be detected separately from the genus *Glaciecola* in the community analysis, likely due to the short sequence length used, but *Glaciecola* was present in both the 500 and 515a cores (Eronen-Rasimus *et al*. 2017). Our results together with those of Eronen-Rasimus *et al*. (2017) support the notion that bacteriophages of these predominant bacteria may be abundant in the viral community, resulting in increased opportunities of isolating them.

**Phage-host interactions**

We tested the sensitivity of 48 isolated bacterial strains, representing nine different genera, to our phages. PANV1 and PANV2 were able to infect several closely related *Paraglaciecola* strains with different EOPs, but not all the strains (Figure 1). This may have been the result of an arms race in which bacterial strains evolve to inhibit phage infection, leading to diversification of bacterial strains (Thingstad *et al*. 2014). Since PANV1 and PANV2 infected different strains, this may have resulted in two different phage-host coevolution lineages.

Phage OANV1 was able to infect bacterial strains from two different classes: Alphaproteobacteria (*Octadecabacter*) and Gammaproteobacteria (*Paraglaciecola*, Figure 1). Still, it was able to infect only three of the *Octadecabacter* strains with

For Peer Review


ScholarOne Support 1-434/964-4100

identical 16S rRNA gene sequences. These phages can seemingly be strain-specific in their host recognition, even if they can infect bacteria across classes. Sea-ice phage isolates have very narrow host ranges (Borriss *et al*. 2003; Luhtanen *et al*. 2014), but based on the genomic data, cold-active phages may have broader host ranges than mesophiles (Colangelo-Lillis and Deming 2013). Myoviruses are often considered to have broader host ranges than siphoviruses and podoviruses (Suttle 2005). However, in this study, myovirus PANV1 and siphovirus PANV2 showed similar host ranges and were able to infect only closely related hosts, whereas siphovirus OANV1 was able to infect strains from two different classes. When phage host ranges are experimentally studied, the number of cultivable bacterial isolates limits the tests, and consequently the results cannot reveal the complete host range spectrum in the environment. However, our results indicate that with the strain specificity observed, the phages may be able to control the bacterial community composition, as proposed earlier based on observation in the environment (Maranger *et al*. 1994), theory (Thingstad *et al*. 2014), and experimentation (Middelboe *et al*. 2001). Since viruses need their hosts to replicate and produce progeny, their activity is directed to the active part of the bacterial community.

**Purification and characterization of phages**

The purification process was optimized for all phages separately. Purification analysis revealed that a significant amount of impurities and host-derived complexes were separated, allowing us to obtain a light-scattering zone comprising infectious, highly purified viruses (Figure 2). The individual protein patterns of the isolated phages (Figure 2) and the specific infectivities calculated (Table 4) showed that each isolated phage was different and that the purification of the virus particles was successful. Efficient purification made it possible to study individual phages in more detail. Detailed TEM observations verified that all the phages isolated were icosahedral tailed phages (Figure 3). Sudan Black staining of the

For Peer Review


ScholarOne Support 1-434/964-4100

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and Triton-X treatment of the virus particles indicated that the phages do not have a structural lipid component, which is in accordance with the virus morphologies observed. The virus capsid diameters (~50–71 nm; Table 3) are similar to those in the most abundant VLP size groups (50–70 nm or <110 nm) reported in Arctic and Antarctic sea ice (Maranger *et al*. 1994; Gowing *et al*. 2004). In three cases, the morphology of the virus tails was reliably identified, suggesting that PANV1 is a myovirus, whereas PANV2 and OANV1 are siphoviruses (Figure 3) resembling the dsDNA bacteriophages belonging to the order *Caudovirales*. However, the tail of bacteriophage OANV2 was considerably difficult to detect. We propose that OANV2 is a podophage with a short noncontractile tail. Icosahedral tailed viruses from the order *Caudovirales* were previously the most often isolated virus types from sea ice (Borriss *et al*. 2003; Luhtanen *et al*. 2014), although a filamentous virus from the order *Inoviridae* has also been isolated (Yu *et al*. 2015).

**Temperature**

The temperatures used here for isolation, cultivation, and tests (Table 5) for both bacteria and phages were warmer than the temperatures in the sea-ice brine (-1.7 °C down to -12.6 °C; Tison *et al*. 2017), due to methodological limitations. It is still evident that the bacteria and phages were cold-adapted, since the bacterial strains were able to grow at 0 °C, but not at 20 °C (Morita 1975), and the phages were capable of infecting and producing progeny at ≤ 4 °C (Wells and Deming 2006b; Table 5). In addition, psychrophilic bacteria from sea ice can be active even at -20 °C (Junge *et al*. 2004), and cold-active phages can be productive at temperatures from 8 °C to -6 °C (Wells and Deming 2006b) or even at -12 °C (Wells and Deming 2006c).

The phages seemed to retain their infectivity well at cold temperatures, since the infectivity of the lysates was stable for several months at 4 °C and at -80 °C

For Peer Review


ScholarOne Support 1-434/964-4100

(supplemented with 15% glycerol), consistent with a previous study (Wells and Deming 2006c). Virus plaques were formed only at temperatures about 10 degrees lower than their host could tolerate, indicating that temperature controlled the infections, as shown in previous studies on sea-ice phage-host isolates (Borriss *et al*. 2003; Luhtanen *et al*. 2014) and other cold-adapted phage-host systems (Wells and Deming 2006b). The virus-receptor molecules may only have been induced at cold temperatures, as reported previously in *Yersinia enterocolitica* infections (Leon-Velarde *et al*. 2016), indicating that the receptors may be associated with the host´s cryoprotection mechanisms. The structure of the phage-receptor molecules could also have changed with rising temperatures, which can inhibit the infection, or the bacterial resistance mechanisms could have been activated at higher temperatures. The phage particles themselves remained infectious and were able to tolerate the temperature of the warm top-layer agar (~43 °C) used for the plaque assay.

**Abundance of virus-like particles and virus-to-bacteria ratios in Antarctic winter-sea ice**

The VLP abundance in Antarctic winter-sea ice ranged from ~$10^5$ to $10^6$ $ml^{-1}$ in bulk ice (Table 1). The highest abundances were measured at stations 500 and 515 ($0.7\times10^6$–$4.9\times10^6$ $ml^{-1}$ in bulk ice). The lower range of our dataset is comparable to the values measured earlier from Antarctic winter-sea ice (~$10^5$ $ml^{-1}$ of bulk ice; Paterson and Laybourn-Parry 2012), whereas the highest abundances were similar to those in Arctic spring blooms and Antarctic late autumn and summer sea ice (~$10^6$–$10^8$ $ml^{-1}$ of bulk ice; Maranger *et al*. 1994; Gowing *et al*. 2002; 2004, respectively). The high VLP concentrations at stations 500 and 515 may be explained by the high bacterial abundance (Eronen-Rasimus *et al*. 2017; Table 1) and bacterial production (measured as thymidine incorporation; Eronen-Rasimus *et al*. 2017) observed, which were positively correlated with the high chl-*a* concentrations (up to 113.2 mg $l^{-1}$ in bulk ice; Eronen-Rasimus *et al*. 2017; Tison


ScholarOne Support 1-434/964-4100
For Peer Review

*et al*. 2017). Positive correlation of chl-*a* with bacterial and VLP abundances was reported in Antarctic sea ice during spring and summer ice-algal blooms (Maranger *et al*. 1994; Gowing *et al*. 2004). Our results suggest that if the chl-*a* concentrations and consequent bacterial abundance and activity are high, viruses may be abundant and likely active in winter-sea ice. The high VLPs also indicate that the viral winter-sea-ice community was surprisingly dynamic, considering the season.

The VBR range of 0.7–13.4 (mean 5.3) corresponds to those measured previously in Antarctic winter-sea ice (1–20.8; Paterson and Laybourn-Parry 2012). The highest VBRs (Table 1) were found at first-year ice-station 503 with low bacterial abundance and activity (Table 1; see bacterial production in Eronen-Rasimus *et al*. 2017), while the lowest VBRs were detected at young second-year ice-station 515 (Table 1) with the highest bacterial production and abundance (Eronen-Rasimus *et al*. 2017; Table 1). The high VBR in the low-activity community may have resulted from induction of lysogenic viruses during freezing and preservation of the virus particles in sea-ice brine, similarly to young ice in the Arctic (Collins and Deming 2011). The decreasing VBR together with increasing VLP, bacterial abundances, and bacterial activity were also detected during the algal spring bloom (Maranger *et al*. 1994). The low VBR in the active community may have resulted from change in the bacterial community composition, so that the majority of the bacteria could have developed resistance against the phages. Alternatively, the host bacterial activity may have been decreased, which could have lowered the viral production, possibly because the phages may have lysogenized, i.e. become prophages (Maranger *et al*. 1994).

In conclusion, four phage-host systems were isolated from both first- and second-year winter-sea ice from the Weddell Sea, Antarctica. The phages seemed to be bacterial strain-specific, but some were able to infect several related bacterial

For Peer Review


ScholarOne Support 1-434/964-4100

strains and one from second-year ice even across classes. The phages were able to retain their infectivity for lengthy periods under cold conditions and infected their host bacteria only in the hosts' lower growth temperature ranges, suggesting that they are cold-active. The VLP counts suggest that the viral community may also be dynamic in winter-sea ice if their hosts are active.

**Funding**

This work was supported by the Walter and Andrée de Nottbeck Foundation (AML, EER, JMR), Onni Talas Foundation (AML), the Academy Professor (Academy of Finland) funding grants 283072 and 255342 (DHB) and the Belgian Science Policy (Bigsouth project, SD/CA/05). BD is a research associate at the F.R.S-FNRS.

**Acknowledgements**

The authors acknowledge the support and use of resources of Instruct, a Landmark ESFRI project. We thank the University of Helsinki and Academy of Finland (grant number 1306833) for support provided to EU ESFRI Instruct Centre for Virus and Macromolecular Complex Production (ICVIR) used in this study. We also acknowledge the Electron Microscopy Unit in the Institute of Biotechnology, University of Helsinki and the Finnish Environment Institute, Marine Research Centre and Finnish Marine Research Infrastructure (FINMARI) for providing the laboratory infrastructure.We also want to thank the Finnish Antarctic Research Program FINNARP (especially Mika Kalakoski and Eivor Lahtinen) for logistic and financial support with cargo and travel expenses, and the Alfred Wegener Institute for Polar and Marine Research, leading researcher Peter Lemke, the captain, crew, and the other participants in the AWECS expedition. We thank Sari Korhonen for skilled technical assistance in virus production and purification, Christiane Uhlig for providing the MOX medium plates, and Harri Kuosa and Daniel Delille for useful comments on the manuscript.

For Peer Review

## Conflict of Interest

The authors declare no conflict of interest.

## References

Anderson NG, Harris WW, Barber AA *et al.* Separation of subcellular components and viruses by combined rate- and isopycnic-zonal centrifugation. *J Natl Cancer Inst Monogr* 1966;**21**:253–83.

Arrigo KR, Mock T, Lizotte MP. Primary producers and sea ice. In: Thomas DN, Dieckmann GS (eds). *Sea Ice.* 2nd ed. Oxford: John Wiley & Sons, Ltd., 2010, 283–325.

Arrigo KR, Thomas DN. Large scale importance of sea ice biology in the Southern Ocean. *Antarct Sci* 2004;**16**:471–86.

Bamford JK, Bamford DH. Large-scale purification of membrane-containing bacteriophage PRD1 and its subviral particles. *Virology* 1991;**181**:348–52.

Borriss M, Helmke E, Hanschke R *et al.* Isolation and characterization of marine psychrophilic phage-host systems from Arctic sea ice. *Extremophiles* 2003;**7**:377–84.

Borriss M, Lombardot T, Glöckner FO *et al.* Genome and proteome characterization of the psychrophilic *Flavobacterium* bacteriophage 11b. *Extremophiles* 2007;**11**:95–104.

Boulanger PA, Puvion F. Large-scale preparation of soluble adenovirus hexon, penton and fiber antigens in highly purified form. *Eur J Biochem* 1973;**39**:37–42.

Bowman JP, McCammon SA, Brown MV *et al.* Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Appl Environ Microb* 1997;**63**:3068–78.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;**72**:248–54.

For Peer Review



ScholarOne Support 1-434/964-4100

Brinkmeyer R, Knittel K, Jurgens J *et al.* Diversity and structure of bacterial communities in Arctic versus Antarctic pack ice. *Appl Environ Microb* 2003;**69**:6610–9.

Brussaard CPD, Payet JP, Winter C *et al.* Quantification of Aquatic Viruses by Flow Cytometry. In: Wilhelm S, Weinbauer M, Suttle C (eds.). *Manual of Aquatic Viral Ecology*. The American society of limnology and Oceanography, Inc, 2010:102–109.

Burgess RR. Chapter 20 - Protein Precipitation Techniques. *Method Enzymol*. Vol 463. 1st ed. Elsevier Inc., 2009, 331–42.

Colangelo-Lillis JR, Deming JW. Genomic analysis of cold-active Colwelliaphage 9A and psychrophilic phage-host interactions. *Extremophiles* 2013;**17**:99–114.

Collins RE, Deming JW. Abundant dissolved genetic material in Arctic sea ice Part II: Viral dynamics during autumn freeze-up. *Polar Biol* 2011;**34**:1831–41.

Deming JW, Collins RE. Sea ice as a habitat for bacteria, archaea and viruses. In: Thomas DN (ed.). *Sea Ice*. 3rd ed. Oxford: John Wiley & Sons, Ltd., 2017, 326–351.

Dieckmann GS, Hellmer H. The importance of sea ice: an overview. In: Thomas DN, Dieckmann GS (eds). *Sea Ice*. 2nd ed. Oxford: John Wiley & Sons, Ltd., 2010, 1–22.

Edwards U, Rogall T, Blöcker H *et al.* Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* 1989;**17**:7843–53.

Eronen-Rasimus E, Luhtanen A-M, Rintala J-M *et al.* An active bacterial community linked to high chl-a concentrations in Antarctic winter-pack ice and evidence for the development of an anaerobic sea-ice bacterial community. *ISME J* 2017;**63**:3068. DOI: 10.1038/ismej.2017.96.

Forterre P, Soler N, Krupovic M *et al.* Fake virus particles generated by fluorescence microscopy. *Trends Microbiol* 2013;**21**:1–5.

For Peer Review



ScholarOne Support 1-434/964-4100

669 Fuhrman JA. Marine viruses and their biogeochemical and ecological effects. *Nature*
670 1999;**399**:541–8.

671 Gowing MM, Garrison DL, Gibson AH *et al.* Bacterial and viral abundance in Ross Sea
672 summer pack ice communities. *Mar Ecol Prog Ser* 2004;**279**:3–12.

673 Gowing MM, Riggs BE, Garrison DL *et al.* Large viruses in Ross Sea late autumn pack
674 ice habitats. *Mar Ecol Prog Ser* 2002;**241**:1–11.

675 Helmke E, Weyland H. Bacteria in sea ice and underlying water of the eastern Weddell
676 Sea in midwinter. *Mar Ecol Prog Ser* 1995;**117**:269–87.

677 Huston AL, Krieger-Brockett BB, Deming JW. Remarkably low temperature optima for
678 extracellular enzyme activity from Arctic bacteria and sea ice. *Environ Microbiol*
679 2000;**2**:383–8.

680 Junge K, Eicken H, Deming JW. Bacterial Activity at -2 to -20 °C in Arctic Wintertime
681 Sea Ice. *Appl Environ Microbiol* 2004;**70**:550–7.

682 Krupovic M, Dutilh BE, Adriaenssens EM *et al.* Taxonomy of prokaryotic viruses:
683 update from the ICTV bacterial and archaeal viruses subcommittee. *Arch Virol*
684 2016;**161**:1095–9.

685 Lane DJ, Pace B, Olsen GJ *et al.* Rapid determination of 16S ribosomal RNA sequences
686 for phylogenetic analyses. *Proc Natl Acad Sci* 1985;**82**:6955–9.

687 Lannuzel D, De Jong J, Schoemann V *et al.* Development of a sampling and flow
688 injection analysis technique for iron determination in the sea ice environment. *Anal Chim*
689 *Acta* 2006;**556**:476–483.

690 Lawrence JE, Steward GF. Purification of Viruses by Centrifugation. In: Wilhelm S,
691 Weinbauer M, Suttle C (eds.). *Manual of Aquatic Viral Ecology*. The American society
692 of limnology and Oceanography, Inc, 2010:166–181.

For Peer Review


ScholarOne Support 1-434/964-4100

Leon-Velarde CG, Happonen L, Pajunen M *et al. Yersinia enterocolitica*-specific infection by bacteriophages TG1 and ϕR1-RT is dependent on temperature-regulated expression of the phage host receptor OmpF. *Appl Environ Microbiol* 2016;**82**:5340–53.

Leturnic I, Bork P. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 2007;**23**:127–128.

Luhtanen A-M, Eronen-Rasimus E, Kaartokallio H *et al.* Isolation and characterization of phage-host systems from the Baltic Sea ice. *Extremophiles* 2014;**18**:121–30.

Maranger R, Bird DF. Viral abundance in aquatic systems: a comparison between marine and fresh waters. *Mar Ecol Prog Ser* 1995;**121**: 217–226.

Maranger R, Bird D, Juniper S. Viral and bacterial dynamics in Arctic sea ice during the spring algal bloom near Resolute, N. W. T., Canada. *Mar Ecol Prog Ser* 1994;**111**:121–7.

Marchant H, Davidson A, Wright S *et al.* The distribution and abundance of viruses in the Southern Ocean during spring. *Antarct Sci* 2000;**12**:414–7.

Meiners K, Krembs C, Gradinger R. Exopolymer particles: microbial hotspots of enhanced bacterial activity in Arctic fast ice (Chukchi Sea). *Aquat Microb Ecol* 2008;**52**:195–207.

Middelboe M, Hagström Å, Blackburn N *et al.* Effects of bacteriophages on the population dynamics of four strains of pelagic marine bacteria. *Microb Ecol* 2001;**42**:395–406.

Middelboe M, Riemann L, Steward GF *et al.* Virus-induced transfer of organic carbon between marine bacteria in a model community. *Aquat Microb Ecol* 2003;**33**:1–10.

Mock T, Thomas DN. Recent advances in sea-ice microbiology. *Environ Microbiol* 2005;**7**:605–19.

Morita RY. Psychrophilic bacteria. *Bacteriol Rev* 1975;**39**:144–67.

For Peer Review

Olkkonen VM, Bamford DH. Quantitation of the adsorption and penetration stages of bacteriophage ϕ6 infection. *Virology* 1989;**171**:229–238.

Paterson H, Laybourn-Parry J. Antarctic sea ice viral dynamics over an annual cycle. *Polar Biol* 2012;**35**:491–7.

Proctor LM, Fuhrman JA. Viral mortality of marine bacteria and cyanobacteria. *Nature* 1990;**343**:60–2.

Pruesse E, Peplies J, Glockner FO. SINA: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* 2012;**28**:1823–9.

Rintala J-M, Piiparinen J, Blomster J *et al.* Fast direct melting of brackish sea-ice samples results in biologically more accurate results than slow buffered melting. *Polar Biol* 2014;**37**:1811–22.

Sait L, Galic M, Strugnell RA *et al.* Secretory Antibodies Do Not Affect the Composition of the Bacterial Microbiota in the Terminal Ileum of 10-Week-Old Mice. *Appl Environ Microbiol* 2003;**69**:2100–9.

Senčilo A, Luhtanen A-M, Saarijärvi M *et al.* Cold-active bacteriophages from the Baltic Sea ice have diverse genomes and virus-host interactions. *Environ Microbiol* 2015;**17**:3628–41.

Shivaji S, Reddy GS. Phylogenetic analyses of the genus *Glaciecola*: emended description of the genus *Glaciecola*, transfer of *Glaciecola mesophila*, *G. agarilytica*, *G. aquimarina*, *G. arctica*, *G. chathamensis*, *G. polaris* and *G. psychrophila* to the genus *Paraglaciecola* gen. nov. as *Paraglaciecola mesophila* comb. nov., *P. agarilytica* comb. nov., *P. aquimarina* comb. nov., *P. arctica* comb. nov., *P. chathamensis* comb. nov., *P. polaris* comb. nov. and *P. psychrophila* comb. nov., and description of *Paraglaciecola oceanifecundans* sp. nov., isolated from the Southern Ocean. *Inter J Syst Evol Micr* 2014;**64**:3264–75.

Soler N, Krupovic M, Marguet E *et al.* Membrane vesicles in natural environments: a major challenge in viral ecology. *ISME J* 2015;**9**:793–6.

For Peer Review



ScholarOne Support 1-434/964-4100

Stamatakis A. RaxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014;**30**:1312–1313.

Suttle C. Viruses in the sea. *Nature* 2005;**437**:356–61.

Suttle CA. Marine viruses – major players in the global ecosystem. *Nat Rev Micro* 2007;**5**:801–12.

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

Thingstad TF, Vage S, Storesund JE *et al.* A theoretical analysis of how strain-specific viruses can control microbial species diversity. *Proc Natl Acad Sci USA* 2014;**111**:7813–8.

Thomas DN, Dieckmann GS. Antarctic sea ice – a habitat for extremophiles. *Science* 2002;**295**:641–4.

Tison J-L, Schwegmann S, Dieckmann G *et al*. Biogeochemical impact of snow cover and cyclonic intrusions on the winter Weddell Sea ice pack. *J Geophys Res Oceans* 2017. DOI: 10.1002/2017jc013288.

Weinbauer MG. Ecology of prokaryotic viruses. *FEMS Microbiol Rev* 2004;**28**:127–81.

Wells L, Deming J. Modelled and measured dynamics of viruses in Arctic winter sea-ice brines. *Environ Microbiol* 2006a;**8**:1115–21.

Wells LE, Deming JW. Characterization of a cold-active bacteriophage on two psychrophilic marine hosts. *Aquat Microb Ecol* 2006b;**45**:15–29.

Wells LE, Deming JW. Effects of temperature, salinity and clay particles on inactivation and decay of cold-active marine Bacteriophage 9A. *Aquat Microb Ecol* 2006c;**45**:31–9.

Wommack KE, Colwell RR. Virioplankton: Viruses in Aquatic Ecosystems. *Microbiol Mol Biol R* 2000;**64**:69–114.

For Peer Review

768 Yoon SH, Ha S-M, Kwon S *et al*. Introducing EzBioCloud: a taxonomically united
769 database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol*
770 *Microbiol* 2017;**67**:1613–1617.

771 Yu Z-C, Chen X-L, Shen Q-T *et al.* Filamentous phages prevalent in Pseudoalteromonas
772 spp. confer properties advantageous to host survival in Arctic sea ice. *ISME J*
773 2015;**9**:871–81.

For Peer Review


ScholarOne Support 1-434/964-4100

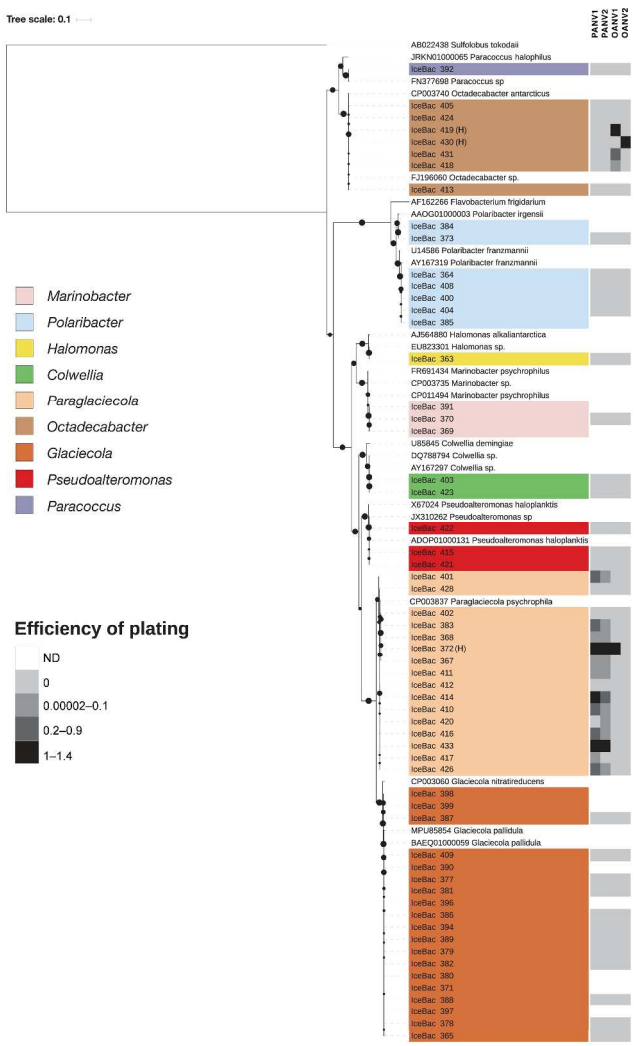



Figure 1 Bootstrapped (1000) phylogenetic maximum-likelihood tree of the 16S rRNA gene sequences of the bacterial strains isolated from Antarctic sea ice. The bootstrap values (> 50%) are shown with black circles. The Sulfolobus tokodaii sequence (AB022438) was used as an outgroup in the alignment. The strains are colored at the genus level (see the color key), based on their classification with SILVA Incremental Aligner (SINA) (version 1.2.11, minimum identity: 0.8, Pruesse et al., 2012). The sensitivities of the bacterial strains to isolated phages are shown as efficiency of plating (EOP; on right in gray scale). For the original host (marked by H), the EOP was set to a value of 1 . The strains used as references or that do not form a lawn on solid growth media were not tested for EOP (marked with white). The scale bar indicates nucleotide substitutions per position.

421x620mm (600 x 600 DPI)

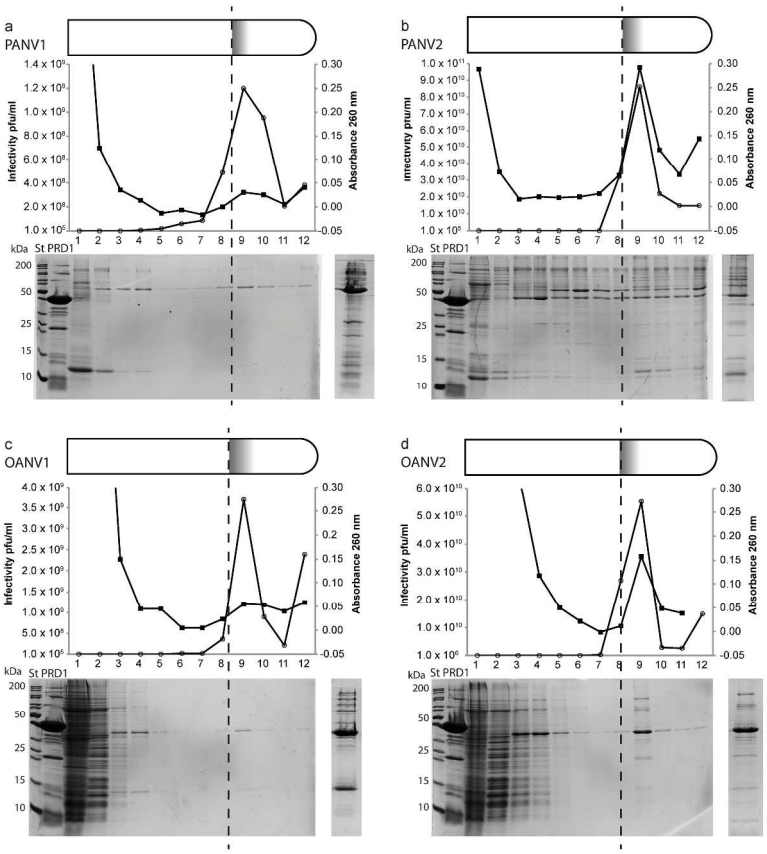
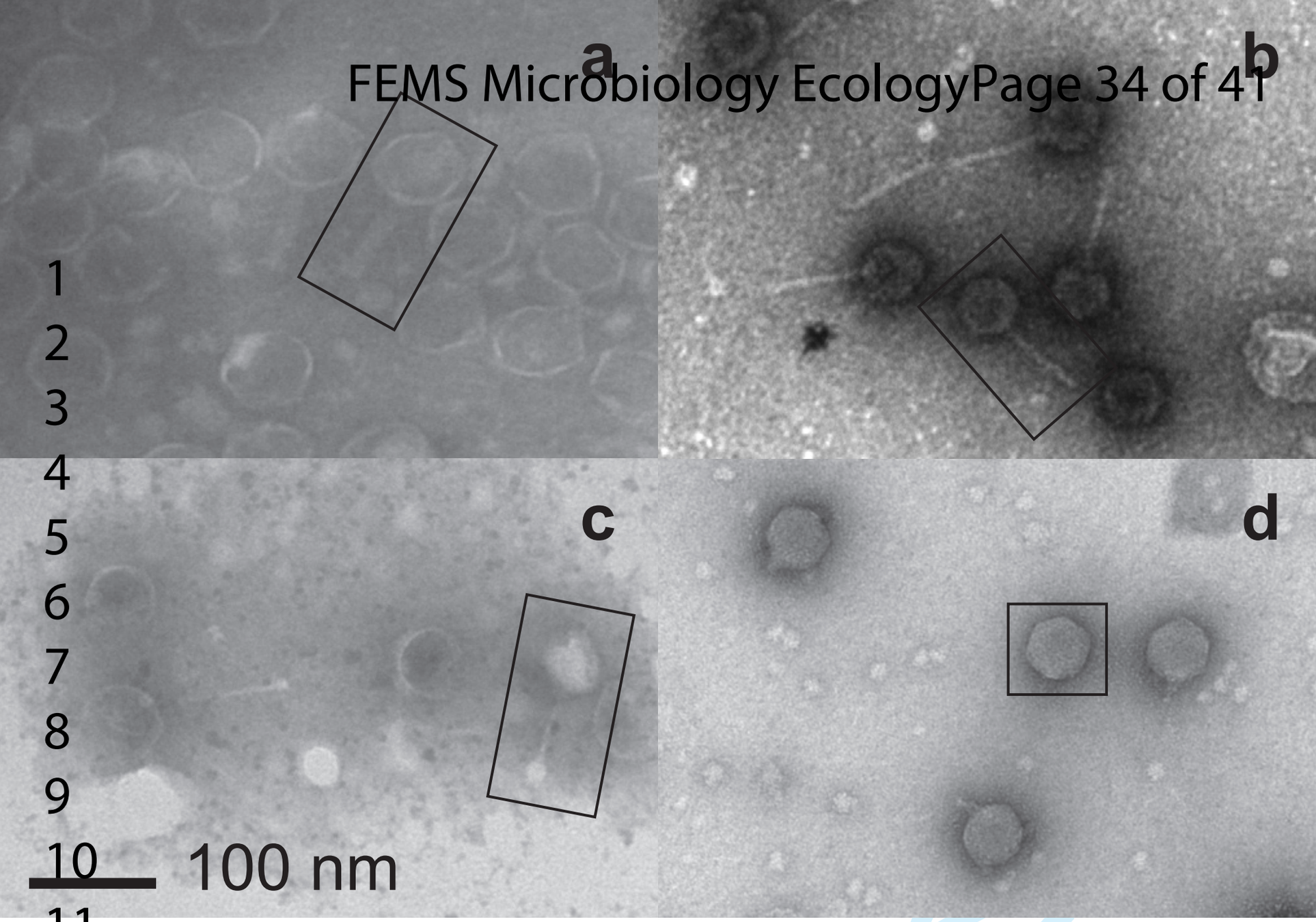



Figure 2 Purification of the phages by rate-zonal centrifugation in sucrose. (a) bacteriophage PANV1, (b) bacteriophage PANV2, (c) bacteriophage OANV1, (d) bacteriophage OANV2. (a–d) Top: position of the light-scattering zone (gray) in the sucrose gradient tubes. Middle: Absorbance (closed squares) and infectivity (open circles) of the 12 sucrose gradient fractions in which the top fraction is marked as 1. Bottom: Protein content of the 12 gradient fractions analyzed with SDS-PAGE and Coomassie Blue staining. The protein patterns of the final biochemically purified and concentrated phages are shown on the right. St = molecular mass marker; PRD1 = purified phage PRD1 used as a control. The dashed line marks the position of the upper edge of the light-scattering virus zone. pfu = plaque-forming unit.

279x373mm (300 x 300 DPI)




ScholarOne Support 1-434/964-4100

## Figure legends

**Figure 1** Bootstrapped (1000) phylogenetic maximum-likelihood tree of the 16S rRNA gene sequences of the bacterial strains isolated from Antarctic sea ice. The bootstrap values (> 50%) are shown with black circles. The *Sulfolobus tokodaii* sequence (AB022438) was used as an outgroup in the alignment. The strains are colored at the genus level (see the color key), based on their classification with SILVA Incremental Aligner (SINA) (version 1.2.11, minimum identity: 0.8, Pruesse *et al.*, 2012). The sensitivities of the bacterial strains to isolated phages are shown as efficiency of plating (EOP; on right in gray scale). For the original host (marked by H), the EOP was set to a value of 1 . The strains used as references or that do not form a lawn on solid growth media were not tested for EOP (marked with white). The scale bar indicates nucleotide substitutions per position.

**Figure 2** Purification of the phages by rate-zonal centrifugation in sucrose. **(a)** bacteriophage PANV1, **(b)** bacteriophage PANV2, **(c)** bacteriophage OANV1, **(d)** bacteriophage OANV2. **(a–d) Top**: position of the light-scattering zone (gray) in the sucrose gradient tubes. **Middle:** Absorbance (closed squares) and infectivity (open circles) of the 12 sucrose gradient fractions in which the top fraction is marked as 1. **Bottom:** Protein content of the 12 gradient fractions analyzed with SDS-PAGE and Coomassie Blue staining. The protein patterns of the final biochemically purified and concentrated phages are shown on the right. St = molecular mass marker; PRD1 = purified phage PRD1 used as a control. The dashed line marks the position of the upper edge of the light-scattering virus zone. pfu = plaque-forming unit.

**Figure 3** Transmission electron micrographs of the purified and negatively stained phages. **(a)** PANV1, **(b)** PANV2, **(c)** OANV1, and **(d)** OANV2.

For Peer Review


ScholarOne Support 1-434/964-4100

Table 1. VLP and bacterial abundances and virus-to-bacteria ratios (VBRs) in the different layers of ice cores from all the AWECS sampling stations.

| Station | Date | Latitude | Longitude | Ice depth (cm) from air-ice interface | | VLP x $10^5$/ml in bulk ice[b] | Bacteria x $10^5$/ml in bulk ice[b,c] | VBR VLP/Bacteria |
|---|---|---|---|---|---|---|---|---|
| | | | | Core I | Core II | | | |
| **486**[a] | 6/17/2013 | -61.526 | -0.086 | 0–6 | – | 1.90 | 0.56 | 3.4 |
| **488**[a] | 6/18/2013 | -62.928 | -0.006 | 0–15 | – | 4.90 | 0.56 | 8.8 |
| | | | | 15–20 | – | 2.60 | 0.81 | 3.2 |
| | | | | 20–35 | – | 6.80 | 1.20 | 5.7 |
| **489**[a] | 6/19/2013 | -63.901 | -0.031 | 0–15 | – | 6.50 | 1.20 | 5.4 |
| | | | | 15–22 | – | 4.90 | 1.30 | 3.8 |
| | | | | 22–37 | – | 5.80 | 0.85 | 6.8 |
| **493** | 6/21/2013 | -66.44 | 0.122 | 0–15 | 0–15 | 13.00 | 3.40 | 3.8 |
| | | | | 15–46 | 15–38 | 9.30 | 3.80 | 2.4 |
| | | | | 46–61 | 38–53 | 9.30 | 1.80 | 5.2 |
| **496** | 6/24/2013 | -67.466 | -0.021 | 0–15 | 0–15 | 4.70 | 1.30 | 3.6 |
| | | | | 15–45 | 15–57 | 3.90 | 2.70 | 1.4 |
| | | | | 45–60 | 57–72 | 5.90 | 2.50 | 2.4 |
| **500** | 7/3/2013 | -67.949 | -6.658 | 0–15 | 0–15 | 16.00 | 2.60 | 6.2 |
| | | | | 15–35 | 15–35 | 12.00 | 3.60 | 3.3 |
| | | | | 35–55 | 35–52 | 46.00 | 4.00 | 11.5 |
| | | | | 55–75 | 52–72 | 20.00 | 3.40 | 5.9 |
| | | | | 75–90 | 72–87 | 9.70 | 2.70 | 3.6 |
| **503** | 7/8/2013 | -67.187 | -13.224 | 0–15 | 0–15 | 8.70 | 0.65 | 13.4 |
| | | | | 15–25 | 15–25 | 7.50 | 0.79 | 9.5 |
| | | | | 25–37 | 25–36 | 9.20 | 0.81 | 11.4 |
| | | | | 37–47 | 36–46 | 10.00 | 0.80 | 12.5 |
| | | | | 47–62 | 46–61 | 6.70 | 0.56 | 12.0 |
| **506** | 7/11/2013 | -67.19 | -23.042 | 0–15 | 0–15 | 3.90 | 0.51 | 7.6 |
| | | | | 15–34 | 15–30 | 5.50 | 0.80 | 6.9 |
| | | | | 34–49 | 30–45 | ND | ND | ND |
| **515**[a] | 7/26/2013 | -63.456 | -51.308 | 0-15 | – | 6.90 | 3.60 | 1.9 |
| | | | | 15-45 | – | 9.30 | 6.00 | 1.6 |
| | | | | 45-75 | – | 21.00 | 7.30 | 2.9 |
| | | | | 75-104 | – | 40.00 | 24.00 | 1.7 |
| | | | | 104-134 | – | 49.00 | 42.00 | 1.2 |
| | | | | 134-164 | – | 10.00 | 9.50 | 1.1 |
| | | | | 164-179 | – | 8.90 | 12.00 | 0.7 |
| **517** | 7/30/2013 | -63.509 | -51.112 | 0–15 | 0–15 | 5.70 | 0.60 | 9.5 |
| | | | | 15–30 | 15–30 | 4.60 | 0.87 | 5.3 |
| | | | | 30–43 | 30–43 | 2.20 | 0.71 | 3.1 |
| | | | | 43–58 | 43–58 | 2.40 | 1.30 | 1.8 |
| | | | | 58–73 | 58–73 | 5.80 | 1.40 | 4.1 |

[a] Only one ice core sampled

[b] Analysed from combined layers of the sibling cores I and II, when two cores were collected.

[c] Bacterial abundances are also published in Eronen-Rasimus et al. (2017)

VLP = virus-like particle, AWECS = Antarctic Winter Ecosystem Climate Study, ND = not determined

For Peer Review

Table 2. Bacterial strains isolated in this study from the ice core 515a.

| Isolation depth from air-ice interface (cm) | Isolation media[a] | Bacterial strain | Closest match genus[b] | Identity % at genus level | Accession number | Used for virus isolation |
|---|---|---|---|---|---|---|
| | | | | | | |
| **0-14** | Z | IceBac 363 | *Halomonas* | 100.0 | KY194856 | ✓ |
| **14-28** | Z | IceBac 364 | *Polaribacter* | 99.7 | KY194850 | ✓ |
| **14-28** | Z | IceBac 365 | *Glaciecola* | 99.6 | KY194821 | ✓ |
| **14-28** | Z | IceBac 367 | *Paraglaciecola* | 99.9 | KY194799 | ✓ |
| **14-28** | Z | IceBac 368 | *Paraglaciecola* | 99.9 | KY194805 | ✓ |
| **14-28** | Z | IceBac 369 | *Marinobacter* | 99.3 | KY194841 | |
| **14-28** | Z | IceBac 370 | *Marinobacter* | 99.3 | KY194842 | ✓ |
| **14-28** | Z | IceBac 371 | *Glaciecola* | 99.6 | KY194822 | |
| **14-28** | Z | IceBac 372 | *Paraglaciecola (H)* | 99.9 | KY194800 | ✓ |
| **14-28** | MOX | IceBac 373 | *Polaribacter* | 100.0 | KY194848 | ✓ |
| **14-28** | MOX | IceBac 377 | *Glaciecola* | 99.6 | KY194823 | ✓ |
| **28-42** | Z | IceBac 378 | *Glaciecola* | 99.6 | KY194828 | ✓ |
| **28-42** | Z | IceBac 379 | *Glaciecola* | 99.6 | KY194824 | ✓ |
| **28-42** | Z | IceBac 380 | *Glaciecola* | 99.6 | KY194825 | |
| **28-42** | Z | IceBac 381 | *Glaciecola* | 99.6 | KY194829 | ✓ |
| **28-42** | Z | IceBac 382 | *Glaciecola* | 99.6 | KY194831 | ✓ |
| **28-42** | Z | IceBac 383 | *Paraglaciecola* | 99.9 | KY194801 | ✓ |
| **28-42** | MOX | IceBac 384 | *Polaribacter* | 100.0 | KY194845 | |
| **42-56** | Z | IceBac 385 | *Polaribacter* | 99.7 | KY194844 | |
| **42-56** | Z | IceBac 386 | *Glaciecola* | 99.6 | KY194815 | ✓ |
| **42-56** | Z | IceBac 387 | *Glaciecola* | 99.5 | KY194816 | ✓ |
| **42-56** | Z | IceBac 388 | *Glaciecola* | 99.6 | KY194826 | ✓ |
| **42-56** | Z | IceBac 389 | *Glaciecola* | 99.6 | KY194817 | ✓ |
| **42-56** | Z | IceBac 390 | *Glaciecola* | 99.6 | KY194830 | |
| **42-56** | Z | IceBac 391 | *Marinobacter* | 99.3 | KY194843 | |
| **42-56** | Z | IceBac 392 | *Paracoccus* | 99.9 | KY194857 | ✓ |
| **42-56** | MOX | IceBac 394 | *Glaciecola* | 99.6 | KY194832 | ✓ |
| **56-70** | Z | IceBac 396 | *Glaciecola* | 99.6 | KY194827 | |
| **56-70** | Z | IceBac 397 | *Glaciecola* | 99.6 | KY194818 | |
| **56-70** | Z | IceBac 398 | *Glaciecola* | 99.5 | KY194820 | |
| **56-70** | Z | IceBac 399 | *Glaciecola* | 99.4 | KY194819 | |
| **56-70** | Z | IceBac 400 | *Polaribacter* | 99.7 | KY194849 | ✓ |
| **56-70** | Z | IceBac 401 | *Paraglaciecola* | 100.0 | KY194802 | ✓ |
| **56-70** | Z | IceBac 402 | *Paraglaciecola* | 99.9 | KY194806 | ✓ |
| **70-84** | C | IceBac 403 | *Colwellia* | 99.4 | KY194851 | ✓ |
| **70-84** | Z | IceBac 404 | *Polaribacter* | 99.7 | KY194846 | ✓ |
| **70-84** | Z | IceBac 405 | *Octadecabacter* | 100.0 | KY194834 | ✓ |
| **98-112** | Z | IceBac 408 | *Polaribacter* | 99.7 | KY194847 | ✓ |
| **98-112** | Z | IceBac 409 | *Glaciecola* | 99.6 | KY194833 | ✓ |

For Peer Review

| | | | | | | |
|---|---|---|---|---|---|---|
| **112-126** | C | IceBac 410 | *Paraglaciecola* | 100.0 | KY194813 | ✓ |
| **112-126** | Z | IceBac 411 | *Paraglaciecola* | 100.0 | KY194808 | ✓ |
| **112-126** | Z | IceBac 412 | *Paraglaciecola* | 100.0 | KY194803 | ✓ |
| **112-126** | Z | IceBac 413 | *Octadecabacter* | 100.0 | KY194835 | ✓ |
| **112-126** | Z | IceBac 414 | *Paraglaciecola* | 100.0 | KY194804 | ✓ |
| **126-140** | C | IceBac 415 | *Pseudoalteromonas* | 100.0 | KY194855 | ✓ |
| **126-140** | Z | IceBac 416 | *Paraglaciecola* | 99.9 | KY194807 | ✓ |
| **126-140** | Z | IceBac 417 | *Paraglaciecola* | 100.0 | KY194809 | ✓ |
| **126-140** | Z | IceBac 418 | *Octadecabacter* | 100.0 | KY194836 | ✓ |
| **126-140** | Z | IceBac 419 | *Octadecabacter (H)* | 100.0 | KY194837 | ✓ |
| **140-154** | Z | IceBac 420 | *Paraglaciecola* | 100.0 | KY194810 | ✓ |
| **140-154** | Z | IceBac 421 | *Pseudoalteromonas* | 100.0 | KY194853 | ✓ |
| **140-154** | Z | IceBac 422 | *Pseudoalteromonas* | 100.0 | KY194854 | ✓ |
| **140-154** | Z | IceBac 423 | *Colwellia* | 99.4 | KY194852 | ✓ |
| **140-154** | Z | IceBac 424 | *Octadecabacter* | 100.0 | KY194838 | ✓ |
| **140-154** | MOX | IceBac 426 | *Paraglaciecola* | 100.0 | KY194814 | ✓ |
| **154-166** | Z | IceBac 428 | *Paraglaciecola* | 99.9 | KY194811 | ✓ |
| **154-166** | Z | IceBac 430 | *Octadecabacter (H)* | 100.0 | KY194839 | ✓ |
| **154-166** | Z | IceBac 431 | *Octadecabacter* | 100.0 | KY194840 | ✓ |
| **154-166** | MOX | IceBac 433 | *Paraglaciecola* | 100.0 | KY194812 | ✓ |

[a] Z = Zobell media; MOX= MOX media; C = concentrated Zobell media.

[b] Original isolation hosts of the viruses are marked by (H).

For Peer Review

**Table 3.** Phages isolated in this study.

| Phage | Sampling station | Isolation host | Genus of the host (closest match) | Lysate titer (pfu/ml) | Capsid head diameter (nm)[a] | Tail length (nm)[b] | Morphotype |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| *Paraglaciecola* Antarctic GD virus 1 (PANV1) | 500 | IceBac 372 | *Paraglaciecola* | $1.5 \times 10^{10}$ | 71±7 (n=20) | 58±22 (n=10) | myovirus |
| *Paraglaciecola* Antarctic JLT virus 2 (PANV2) | 500 | IceBac 372 | *Paraglaciecola* | $5.2 \times 10^{11}$ | 52±8 (n=29) | 89±30 (n=10) | siphovirus |
| *Octadecabacter* Antarctic BD virus 1 (OANV1) | 515 | IceBac 419 | *Octadecabacter* | $1.2 \times 10^{10}$ | 50±8 (n=20) | 83±10 (n=10) | siphovirus |
| *Octadecabacter* Antarctic DB virus 2 (OANV2) | 515 | IceBac 430 | *Octadecabacter* | $5.8 \times 10^{9}$ | 53±7 (n=20) | – | podovirus |

[a] average diameter
[b] average length
pfu = plaque-forming unit

For Peer Review

**Table 4.** Recovery of infectious phages during biochemical purification after ammonium sulfate precipitation and rate zonal centrifugation in sucrose combined with concentration step by differential centrifugation.

| | | Total pfus[a] | Recovery of infectivity % | Specific infectivity pfu / mg protein |
|---|---|---|---|---|
| **PANV1** | | | | |
| | Virus lysate | $7.5 \times 10^{12}$ | 100.0 | |
| | 50 % ammonium sulfate precipitate | $4.1 \times 10^{12}$ | 54.7 | |
| | Concentrated virus[b] | $1.5 \times 10^{12}$ | 20.0 | $1.8 \times 10^{12}$ |
| | | | | |
| **PANV2** | | | | |
| | Virus lysate | $3.2 \times 10^{14}$ | 100.0 | |
| | 50 % ammonium sulfate precipitate | $9.6 \times 10^{13}$ | 30.0 | |
| | Concentrated virus[b] | $6.2 \times 10^{13}$ | 19.4 | $8.9 \times 10^{12}$ |
| | | | | |
| **OANV1** | | | | |
| | Virus lysate | $6.0 \times 10^{12}$ | 100.0 | |
| | 80 % ammonium sulfate precipitate | $1.5 \times 10^{12}$ | 25.0 | |
| | Concentrated virus[b] | $5.7 \times 10^{11}$ | 9.5 | $3.8 \times 10^{12}$ |
| | | | | |
| **OANV2** | | | | |
| | Virus lysate | $3.3 \times 10^{12}$ | 100.0 | |
| | 80 % ammonium sulfate precipitate | $1.2 \times 10^{12}$ | 35.7 | |
| | Concentrated virus[b] | $4.4 \times 10^{11}$ | 13.6 | $6.9 \times 10^{12}$ |

[a] calculated per a liter of original lysate

[b] after rate zonal centrifugation in sucrose and concentration by differential centrifugation

pfu = plaque-forming unit

For Peer Review

**Table 5.** Temperature-dependent growth of the bacterial host strains and the phages.

| Host bacterial growth[a] | | | | | |
|---|---|---|---|---|---|
| | | | | | |
| | **0°** | **4°** | **10°** | **15°** | **20°** |
| IceBac 372 | + | + | + | (+) | - |
| IceBac 419 | + | + | + | + | - |
| IceBac 430 | + | + | + | + | - |
| | | | | | |
| Infectivity of the phages[a] | | | | | |
| | | | | | |
| PANV1 | + | + | - | - | ND |
| PANV2 | + | + | - | - | ND |
| OANV1 | + | + | - | - | ND |
| OANV2 | + | + | - | - | ND |

[a] + = producing colonies/plaques; (+) = retarded growth; – = no colonies or plaques produced; ND = not determined.

For Peer Review