Efficient Dilution-to-Extinction isolation of novel virus-host model systems for fastidious heterotrophic bacteria.

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Abstract

Microbes and their associated viruses are key drivers of biogeochemical processes in marine and soil biomes. While viruses of phototrophic cyanobacteria are well-represented in model systems, challenges of isolating marine microbial heterotrophs and their viruses have hampered experimental approaches to quantify the importance of viruses in nutrient recycling. A resurgence in cultivation efforts has improved the availability of fastidious bacteria for hypothesis testing, but this has not been matched by similar efforts to cultivate their associated bacteriophages. Here, we describe a high-throughput method for isolating important virus-host systems for fastidious heterotrophic bacteria that couples advances in culturing of hosts with sequential enrichment and isolation of associated phages. Applied to six monthly samples from the Western English Channel, we first isolated a new SAR11 and three new members of the methylotrophic bacterial clade OM43, and used these as bait to isolate 117 new phages including the first known siphophage infecting SAR11, and the first known phages for OM43. Genomic analyses of 13 novel viruses revealed representatives of three new viral genera, and infection assays showed that SAR11 viruses have ecotype-specific host-ranges. Similar to the abundant human-associated phage ϕCrAss001, infection dynamics within the majority of isolates suggested either prevalent lysogeny, irrespective of detectable associated genes, and/or host phenotypic bistability, with lysis putatively maintained within a susceptible subpopulation. Broader representation of virus-host systems in culture collections and genomic databases will improve both our understanding of virus-host interactions, and accuracy of computational approaches to evaluate ecological patterns from metagenomic data.

Introduction

It is estimated that viral predation kills ~15% of bacterial cells in marine surface water each day [1] and is a major contributor to nutrient recycling via the viral shunt, where marine viruses make cell-bound nutrients available to the neighbouring microbial community through viral lysis of host cells [2, 3]. Viruses are key players in the modulation of carbon fluxes across the oceans (150 Gt/yr), increasing particle aggregation and sinking to depth [2, 4], and accounting for 89% of the variance in carbon export from surface water to the deep ocean [5]. Viruses alter host metabolism through auxiliary metabolic genes (AMGs), increasing and altering the cellular carbon intake of infected cells [6]. Virus-host interactions also increase co-evolutionary rates of both predator and prey via Red Queen dynamics [7, 8]. While recent metagenomic advances have provided major insight into global viral diversity and abundance [9–12], mechanistic understanding of virus-host interactions in ecologically important taxa is reliant on experimental co-culturing of model systems. In cyanobacteria, such systems have shown that viruses increase duration of photosynthetic function [13] and can inhibit CO_2 fixation, providing direct evidence that viruses of abundant phototrophs have a direct and major role in nutrient regulation and global carbon cycling [14]. Furthermore, isolation of novel viral model systems provides complete or near-complete viral genomes with known hosts, critical to the development, ground-truthing and application of computational methods to identify viral genomes in metagenomic data (e.g. VirSorter [15]; VirFinder [16]; MARVEL [17]); quantify boundaries for viral populations [12, 18] and genera (VConTACT2 [19]); understand the importance of AMGs in altering nutrient flux in natural communities [20] and to predict host ranges of uncultured viruses *in situ* (e.g. WIsH [21, 22]).

In comparison to viruses of primary producers, such as cyanophage, which are well represented with model systems and well-studied in the laboratory, virus-host model systems for similarly important and abundant marine heterotrophic bacteria are rare. Available models of viruses infecting heterotrophs are heavily biased towards those with a fast-growing copiotrophic host that grow readily on solid agar, enabling the use of plaque assays to isolate associated viruses. Such systems are not

representative of the vast majority of heterotrophs in nutrient-limited soil and aquatic environments, which are dominated by slow-growing, oligotrophic taxa with few regulatory mechanisms and complex auxotrophies that do not lend themselves to growth on solid or rich medium [23–26]. Advances in Dilution-to-Extinction culturing of ecologically important hosts have enabled the cultivation of many fastidious bacterial taxa that are not amenable to growth on solid media from soil, marine [27, 28] and freshwater environments [29]. Without plaque assays to identify infection and purification of new viral taxa, cultivation of associated viruses is challenging, and exacerbated by the slow growth rates and complex nutrient requirements of their hosts. Paucity of such model systems introduces significant bias in our understanding of viral influence on global carbon biogeochemical cycles. Therefore, it is important that the efforts to isolate heterotrophic bacterial taxa for experimentation and synthetic ecology is matched by efforts to isolate their associated viruses.

Here, we adapted recent advances in Dilution-to-Extinction culturing of hosts [28] and protocols to isolate viruses from liquid media [30] to improve efficiency of recovering novel virus-host systems for fastidious taxa: First, we used sequential enrichment of viruses from natural communities on target hosts to improve rates of viral isolation [31]; Second, we replaced the requirement for time-intensive epifluorescent microscopy with comparative flow cytometry between infected and uninfected hosts to screen for putative viral infection, followed by confirmation using Transmission Electron Microscopy (TEM). We selected the ecologically important, genomically streamlined SAR11 and OM43 heterotrophic marine clades as models for viral isolation. These clades are abundant and important to global carbon biogeochemistry [23, 32–36], but little is known of their associated viral diversity. In the case of viruses infecting SAR11, challenges in assembling abundant and microdiverse genomes from viral metagenomes limit our ability to determine associated host-virus ecology and representatives for *in silico* host prediction [12, 37, 38], particularly for novel species that lack any representation in the databases. This was demonstrated in the successful isolation of the first known pelagiphages by culturing, including the globally dominant HTVC010P, which, prior to

its isolation, was entirely missed in marine viromes [39]. To the best of our knowledge there are no known viruses for OM43, which plays an important role in remineralisation of volatile carbon associated with phytoplankton blooms [32, 34, 40]. As well as their importance in experimentally evaluating the impact of predator-prey dynamics on global marine carbon biogeochemistry, these systems are critical to understand the influence of genome minimalism on host-virus co-evolution. Novel SAR11 and OM43 representatives from the Western English Channel were isolated and used as bait to isolate associated viruses. Initial concentration of viruses in inocula by tangential flow filtration, followed by one to three rounds of sequential enrichment on target hosts in 96-well plates yielded 117 viral isolates from 218 inoculated cultures from seven monthly water samples from September 2018 to July 2019. 94 out of 105 (90%) inoculated SAR11 cultures yielded positive viral infections. 23 out of 113 (20%) inoculated OM43 cultures yielded positive infections. A subsample of positive infections for both clades were subsequently purified and sequenced, revealing the first known siphovirus infecting SAR11 and providing the first known host-virus model for OM43.

Results and Discussion

Isolation of a novel SAR11 strain and three new OM43 strains from the Western English Channel to use as bait for phage isolation

Dilution-to-Extinction culturing for host taxa using natural seawater-based medium on three 96-well plate samples in September 2017 yielded the first SAR11 strain to be isolated from the Western English Channel (named H2P3α) and the first three new OM43 strains (named C6P1, D12P1, H5P1) from the Western English Channel. The full-length 16S rRNA gene of H2P3α was 100% identical to that of the warm water SAR11 ecotype *Pelagibacter bermudensis* HTCC7211 (subclade 1A) and was considered to be a local variant [41] (Supplementary Fig. 1A). All three novel OM43 isolates (named CP61, D12P1, H5P1) were most closely related to *Methylophilales* sp. HTCC2181 (C6P1 96.17%,

D12P1 96.62%, H5P1 97.79% nucleotide identity across the full 16S rRNA gene), a streamlined member of the OM43 clade with a 1.3 Mbp genome, isolated from surface water of the North Eastern Pacific [42] (Supplementary Fig. 1B). The average identity of the 16S rRNA gene of isolates CP61, D12P1 and H5P1 to each other was ~98.46% (Supplementary Table 1), suggesting they are representatives of the same genus [43].

An efficient, low-cost method of isolating new viruses yielded 117 new viral isolates for SAR11 and OM43 taxa

Using the four new hosts and established SAR11 isolates *Pelagibacter ubique* HTCC1062 (cold-water ecotype) and HTCC7211 (warm water ecotype), we developed an optimised viral isolation pipeline (Fig. 1) and applied it to six monthly water samples from the Western English Channel, taken between September 2018 to April 2019 (Supplementary Table 2). Briefly, each month, we inoculated one to two 96-well Teflon plates containing our four new hosts, plus SAR11 isolates HTCC1062 and HTCC7211 at ~10^6 cells per mL with a natural viral community concentrated by tangential flow filtration. Plates were monitored by flow cytometry and growth of putatively infected cultures was compared to those of uninfected controls over the course of ~2 weeks, due to the slow growth rates of SAR11 and OM43 [34, 44]. Additional samples were taken July 2019 to attempt viral isolation on OM43 strains D12P1 and H5P1. In total, out of 218 cultures inoculated with concentrated natural viral communities for initial isolation, 117 viruses were purified and still infective after at least three passages, evidenced by differences in cytograms between cultures inoculated with viruses and control cultures (Supplementary Fig. 2-5), demonstrating an overall efficiency of 53% and an average yield of 18 isolates per sample (Fig. 2). All viral isolations required between one and three rounds of host enrichment (Supplementary Table 3) before changes in host growth curves between infected and uninfected cultures could be observed by flow cytometry, suggesting a minimal viral density is

143 required to identify infected wells using this approach, but this threshold can be reached through
144 successive rounds of enrichment.
145
146 For each sample, all steps of the initial viral isolation process (counting one to two plates for a total
147 of three times) required ~6 hours run time on a flow cytometer. For the subsequent three rounds of
148 purification, one plate was required for each host-sample combination (another ~6 hours of cytometer
149 run time in total for one host-sample combination). Both initial isolation of viruses from
150 environmental water samples and three rounds of purification took ~10 weeks of incubation time in
151 total. Between all steps, our protocol required ~7 hours of handling time per sample. Following three
152 rounds of viral purification, generating sufficiently high viral titres to extract enough DNA for
153 sequencing (approximately two weeks incubation time, and roughly four hours handling time over
154 two days) was the rate-limiting step, and so required subselection of available viruses for sequencing.
155 Future advances in DNA extraction efficiency, reducing DNA input requirements for sequencing
156 and/or automation of viral DNA extraction will enable all isolated viruses to be sequenced. We
157 estimate the cost of isolating a single virus is ~£20 for cultivation, flow cytometry and DNA
158 extraction consumables, and ~£50-100 for genome sequencing at 30-fold coverage required for
159 successful assembly, giving a total cost of £70-120 per sequenced viral isolate, not including the costs
160 for person-hours. Thus, our protocol provides a high-throughput and scalable approach to viral
161 isolation.
162
163 **Novel pelagiphages are ecotype-specific and persist in the community**
164 We hypothesised that the density dependence of predator/prey dynamics would result in greater
165 isolation efficiency of viruses on the cold-water SAR11 ecotype, HTCC1062, during winter months,
166 when this host is more abundant. Contrary to our expectations, the isolation efficiencies (number of
167 successes / number of attempts) were: 80% for HTCC7211, 93% for HTCC1062 and 96.6% for
168 H2P3α, but these were not significantly different (see Supporting Methods: HTCC1062 vs. H2P3α

169 (*P*=0.5, 999 bootstraps); HTCC1062 vs. HTCC7211 (*P=0.18*, 999 bootstraps)). Temperature ranged
170 from 9.3 °C - 14.1 °C from October to April, when isolation was attempted on all strains
171 (Supplementary Table 2). Neither temperature, ecotype nor an ecotype*temperature interaction term
172 were significantly correlated with isolation efficiency based on a generalized linear model using a
173 quasi-binomial error distribution (see Supplementary Methods). One explanation for this result is that
174 host ecotype abundances are constant throughout the year and unaffected by temperature. HTCC1062
175 and HTCC7211 have specific growth rates of ~0.22 and ~0.12 divisions per day at 10 °C, respectively
176 [44], and our new SAR11 isolate from the Western English Channel, H2P3α, showed similar growth
177 rates to HTCC7211 (Supplementary Fig. 6), with specific growth rates of ~0.10, ~0.45 and ~0.84
178 divisions per day at 10, 18 and 25 °C, respectively. Measured lower specific growth rates of warm
179 water ecotypes at winter temperatures, and evidence of global ecotype niche partitioning [45] suggest
180 that uniform ecotype abundance across seasons in the Western English Channel is unlikely, but that
181 slow growth of warm water ecotypes at *in situ* temperatures is possible.
182
183 An alternative explanation is that isolated viruses have a broad host range that encompasses both
184 warm- and cold-water ecotypes. We tested the host range (Table 1) of six pelagiphage population
185 representatives out of the 12 pelagiphages isolated from samples in October 2018 and November
186 2018 (*in situ* water temperature of 14.8 °C and 14.2 °C respectively) across the three SAR11 strains
187 (Table 2). Viruses *Eistla, Eyrgjafa* and *Greip* all infected cold-water ecotype HTCC1062,
188 exclusively, while *Ran* and *Kolga* only infected warm-water ecotypes HTCC7211 and H2P3α.
189 Therefore, our new pelagiphages appear to be broadly ecotype-specific, confirming previous findings
190 [46], with important consequences for their global distribution. *Bylgia* was the only virus that could
191 infect both warm and cold-water ecotypes. Ecotypic specificity at first seems at odds with our ability
192 to isolate viruses on the warm water SAR11 ecotype at *in situ* temperature far below its optimal
193 growth temperature. A possible explanation is that pelagiphages are ecotype-specific, but persist in
194 the water column throughout the year in sufficient densities to be isolated by our enrichment method.

Measured *in situ* temperatures during our sampling period were sufficient to support slow growth of warm-water ecotypes even during winter months, potentially providing sufficient prey to support a population of warm-water ecotype specific phages. If concentration and enrichment of viruses during isolation is sufficient to successfully isolate even low abundance phages then a comprehensive library of representative phage isolates could be generated with relatively modest sampling effort across a few locations.

First methylophages for marine OM43 isolated.

Viral isolation of novel methylophages for the OM43 clade yielded 23 positive infections, with efficiencies ranging from 0% (no viruses isolated on host C6P1) to 45% on H5P1 (Fig. 2). To the best of our knowledge these are the first reported viruses infecting members of the OM43 clade. One explanation for the lower efficiency of isolation of OM43 viruses is simply one of lower host abundance concomitant with lower phage abundance in the viral community, reducing the likelihood of infective viruses coming into contact with susceptible and permissive cells. OM43 are closely associated with metabolism of extracellular substrates from phytoplankton blooms [47], but have low abundance outside of phytoplankton spring blooms [32]. Our water samples were not associated with high *in-situ* fluorescence (used as proxy measurement for phytoplankton), and missed the April 2019 Spring bloom by about two weeks (Supplementary Fig. 7). Therefore, OM43 and their viruses were likely of lower abundance at sampling times, decreasing the success rate of viral isolation from these samples.

New viruses represent novel viral populations and support established ANI cut-offs for ecologically discrete viral ecotypes

Following three rounds of purification through Dilution-to-Extinction, due to the rate-limiting step of extracting viral DNA, we subselected 16 viral isolates based on availability in November 2018 across four different hosts (HTCC1062, HTCC7211, H2P3α and H5P1) for sequencing at >30-fold coverage

(Table 2). Three out of 16 sequenced samples (two from host HTCC7211, one from OM43 host H5P1) failed to assemble into single viral contigs. For 11 of the remaining 13 samples (12 from SAR11 hosts and one from OM43) each individual sequence assembly was identified as a complete viral genome by VirSorter [15] and 95-100% complete using CheckV [48]. OM43 phage *Venkman* possesses a linear but complete genome (Supplementary Table 4). Sequence data yielded a single viral genome (categories 1 or 2, >15kbp) per sample, indicating that our purification process was effective in recovering pure viral isolates.

Viral populations are defined as discrete ecological and evolutionary units that form non-overlapping clouds of sequence space, and average nucleotide identity (ANI) cutoffs of >95% have been proposed from cyanophage studies to delineate viral populations [49]. To evaluate whether our new isolates represented discrete ecological units with respect to other known pelagiphages, pairwise ANI was calculated between the thirteen successfully sequenced viral genomes from this study and 85 other known or putative pelagiphages [37, 46, 50]. Pairwise ANI ranged between 77.5-100%, with a discrete distribution between 96.4-100.0% (Supplementary Fig. 8, Supplementary Table 5). This is in agreement with previous work in cyanophages [49, 51] and thus supports their application to define viral populations beyond cyanophages for viral genomes derived from metagenomic assembly [10, 11]. At proposed ANI cutoffs of 95% over 85% length [18], the new viruses clustered into six viral populations, ranging from singletons to a viral population with four members (Table 2). Phages within the same populations were all isolated from the same source water sample and on the same host, in agreement with their classification as discrete ecological and evolutionary units. Interestingly, viral population membership was exclusive to viruses isolated in this study, with no associated populations containing representatives from either known isolates [39, 46] or fosmid-derived [37] genomes from other studies, including pelagiphage HTVC010P [39], which is ubiquitous and dominant throughout global oceans, including the Western English Channel [12]. Such a distinct separation between populations associated with viruses sampled in this study and all other

pelagiphages, either in culture or assembled from metagenomes, suggests a high degree of viral population diversity remains to be discovered in the Western English Channel and beyond.

Current pelagiphage isolates can be organised into five distinct phylogenetic clades

To evaluate isolate diversity at higher taxonomic organisation, chosen representatives of each of the six viral populations were compared to previous isolates and fosmid-derived phage sequences using three approaches: First, where possible, phylogenetic analysis was performed based on conserved genes; Second, to capture broader relationships and account for genomic mosaicism, raw hypergeometric probability of shared gene content; Finally genomes were organised into ICTV-recognized genera using vConTACT2 (Supplementary Fig. 9), which initially derives viral clusters using a similar hypergeometric approach followed by refinement with Euclidean-distance-based hierarchical clustering to split mismatched, 'lumped' viral clusters [19]. All three approaches were congruent - clustering on probability of shared gene content organising pelagiphage genomes into four main clusters and one singleton genome from this study (*Kolga*) (Fig. 3A-C), which was broadly supported by phylogenetic and vConTACT2 classifications. Cluster A contained 23 members (nine from fosmid-derived contigs [37]; eleven previously isolated pelagiphages [46, 52]), including *Ran*, *Bylgja* and *Eistla* from this study. Cluster B contained two previously isolated pelagiphages, one fosmid-derived contig and *Eyrgjafa* from this study. All viruses in Clusters A and B were assigned to a single viral genus by vConTACT2 that also contained 12 previously isolated pelagiphages [39, 46]. Cluster C only contained fosmid-derived contigs from the Mediterranean [37], with no isolated representatives, making it an important location for future isolation attempts of different hosts and viruses using our method. Cluster D contained eight fosmid-derived contigs, pelagiphage HTVC010P, and a new representative, *Greip*, from this study (Fig. 3B). VConTACT2 split Cluster D, leaving *Greip* and *Kolga* as members of two singleton clusters, suggesting they are the first cultured representatives of novel viral genera.

Kolga - the first siphovirus infecting SAR11

The 25 previously known viral isolates infecting SAR11 comprise 24 podoviruses and one myovirus [39, 46, 53]. Previous cultivation efforts for viruses of SAR11 has not isolated any siphoviruses, nor are any known from viral metagenomic studies. We isolated and sequenced the two viruses *Kolga* and *Aegir* using H2P3α as bait, which to the best of our knowledge are the first known siphoviruses infecting members of the SAR11 clade (Fig. 4A-C). The phages appeared to have a long tail on transmission electron microscopy (TEM) images and are thus morphologically indicative of belonging to the *Siphoviridae* (Fig. 4B). *Aegir* and *Kolga* were members of the same population using a boundary cutoff of 95% ANI over 85% contig length, however, *Aegir* had a length of 18,297 bp compared to 48,659 bp in *Kolga*, therefore we considered *Aegir* to be a partial genome of the same viral population. *Kolga* did not share a significant number of genes with known SAR11 podoviruses (Fig. 3B), and did not cluster with other pelagiphages using a hypergeometric analysis based on gene content. VConTACT2 also grouped *Aegir* and *Kolga* into one cluster without any other known viruses, suggesting it represents a novel viral genus. A number of genes found in *Kolga* were shared with other known siphoviruses, and phylogenetic analysis of concatenated genes shows *Kolga* is most closely related to LK3, a known siphovirus infecting the Betaproteobacterial genus *Bordetella* (Fig. 4C). This suggests that *Kolga* is more closely related to siphoviruses of different hosts compared to podoviruses infecting the same host. 67% of genes encoded by Kolga could not be functionally annotated, and out of all hypothetical genes identified on *Kolga*, only three hypothetical genes were shared with SAR11 podoviruses. In contrast, for on average ~90% of genes without known function identified within our novel SAR11 podoviruses are shared between different pelagiphages. *Kolga* was the 10th most abundant viral isolate in our Western English Channel virome (Fig. 3D). *Kolga* possesses a tail tip J protein (Fig. 4A), often found in phages with long non-contractile tails such as ƛ, where it plays a role in DNA injection during cell entry and tail assembly [54]. *Kolga* also encodes

a small S21 subunit of the 30S ribosomal gene structurally similar to the ones found in HTVC008M, and hosts HTCC7211 and H2P3α, a feature found in numerous myoviruses and sipoviruses [55]. S21 is involved in translation initiation and needed for the binding of mRNA [56]. Virally-encoded S21 genes may provide a competitive advantage for the phage as it could replace cellular S21 and assist in the translation of viral transcripts. *Kolga* may need its S21 gene for shifting the translational frame, as it has been shown that for some members of the Caudovirales the production of tail components is dependent on programmed translational frameshifting [57]. Given the constitutive nature of gene expression in genomically streamlined bacteria [58], genes such as S21 may provide the virus with a mechanism to manipulate host metabolism in the absence of typical promoters and repressors.

Kolga and Greip are the most abundant of the novel viruses in Western English Channel

To evaluate relative abundance of our new isolates in the Western English Channel, we used competitive mapping of metagenomic reads from a Western English Channel virome taken in September 2016 (*in situ* surface water temperature of ~16.1 °C) [12] against existing pelagiphage genomes and those from this study using FastViromeExplorer [59]. This identified HTVC010P as the most abundant virus within the known pelagiphages (536,531 Reads Per Million [RPM - see Supplementary Methods]). Other isolates from this and previous studies recruited 1-3 orders of magnitude fewer reads than HTVC010P (684 - 53,591 RPM), indicating that viral community structures for pelagiphages in the Western English Channel consist of a small number of viral species that are highly abundant, and long tail of lower abundance members (Fig. 3D). *Greip* and *Kolga* were the two most abundant of our new viral isolates in the Western English Channel. Their abundance could be driven in part by the fact that either the *in situ* surface water temperature when the virome sample was taken matches the optimum growth temperature of host HTCC1062 (~0.44 divisions per day at 16 °C) [44], in line with proposed Kill-the-Winner predator/prey dynamics [60], and/or that genomic novelty promotes recruitment of reads closely related taxa that have no other representation

in the reference dataset [39]. Future work will evaluate the global abundance of these and newly sequenced isolates across spatial (Global Ocean Viromes) and temporal gradients (an ongoing time-series project in the Western English Channel) to evaluate their biogeography.

Venkman - the first known virus infecting OM43

Our isolation efforts in the Western English Channel yielded the first cultured bacteriophage infecting the methylotroph OM43 (Fig. 5A-C). Named *Venkman*, in homage to the actor Bill Murray in the film Ghostbusters, this virus has a genome 38,624 bp long (31.9% GC content), encoding genes (Fig. 5A) with similar synteny and function to the siphovirus P19250A (38,562 bp) that infects freshwater *Methylophilales* LD28, which are often considered a freshwater variant of OM43 [61, 62]. Unlike P19250A, TEM images indicated that *Venkman* had a short tail (Fig. 5B) similar to podoviruses, though it is possible that tail structures had been lost during grid preparation. Phylogenetic analysis of concatenated TerL and exonuclease genes further indicated that these genes in *Venkman* are most closely related to other siphoviruses infecting different Proteobacteria (Fig. 5C). However, branch support values were low, despite numerous attempts to refine the tree with different approaches (see Supplementary Methods). We identified a number of common phage proteins such as a capsid protein, terminase, nucleases and tail structural proteins, the remaining 54% of genes were hypothetical. VConTACT2 assigned *Venkman* and P19250 to the same genus-level cluster, therefore *Venkman* may be a marine variant of the freshwater phage P19250. In the Western English Channel viral metagenomes, *Venkman*-like viruses were the second most abundant viruses (148,681 RPM) after HTVC010P (536,531 RPM) identifying them as ecologically important viruses in this coastal microbial community (Fig. 3B).

Unusual host-virus dynamics are prevalent in isolated phages

Viral isolates *Bylgja*, *Eyrgjafa*, *Ran* and *Eistla* (Cluster A and B) are presumed to be temperate phages, with all encoding endonucleases and exonucleases (Supplementary Fig. 10) and clustering by shared protein content with other pelagiphages, such as e.g. HTVC011P and HTVC025P, shown previously to integrate into host genomes (Fig. 3B) [46]. *Eyrgjafa* encodes a tRNA-Leu, that has 85% nucleotide identity over the first 34 bases of the tRNA-Leu of its host HTCC1062, suggesting a putative integration site into the host genome [63]. To date, 16 of the 29 viruses isolated on SAR11 strains have either been shown to be capable of lysogeny, and/or encode genes associated with a temperate infection cycle. In contrast, viruses such as *Greip,* and the abundant HTVC010P in Cluster D (Fig. 3B) do not possess any genes associated with lysogeny, and would therefore presumed to be exclusively lytic. Viruses in Clusters A and B, presumed temperate, were of much lower abundance in the environment compared to *Greip* or HTVC010P in Cluster D which were among the most abundant pelagiphages in the Western English Channel (Fig. 3D), suggesting a possible ecological difference between two groups of viruses with different infectivity strategies.

Interestingly, growth curves of hosts infected with *Greip* and other isolated phages deviated from the expected decay in cell abundance associated with viral lysis and previously observed in isolated pelagiphages [39]. The first pelagiphages HTVC010P and HTVC008M were isolated from the warm waters of the Sargasso Sea, and HTVC011P and HTVC019P were isolated from the colder waters of the Oregon Coast. All four strains were propagated on the cold-water SAR11 ecotype *P. ubique* HTCC1062. In all cases, host density was reduced from ~8×10^6 cells per mL at T_0 to $<10^6$ cells per mL over a 60-72h period, although viruses from warm waters took 17% longer than those from cold water to do so [39], suggesting that suboptimal hosts reduced the rate of infection as shown in cyanophages [64]. In contrast, infection dynamics of our isolates often resulted in host density of infected cultures growing to a steady state, but at a lower cell density than uninfected cells. (Supplementary Video 1), irrespective of cluster or population assignment. Out of 117 viruses isolated in this study, only 16 infections reduced host abundance below their inoculum density of 10^6 cells per

377 mL. In 53 infections, densities of infected cells increased to within an order of magnitude of
378 uninfected cells (Supplementary Fig. 11), but demonstrated clear evidence of viral infection in
379 cytograms, TEMs and subsequent recovery of viral genomes in selected samples.
380
381 Similar patterns of infection were recently reported in the extremely abundant bacteriophage
382 ϕCrAss001 found in the human gut, where infection of exponentially growing cells of *B. intestinalis*
383 919/174 did not result in complete culture lysis, but caused a delay in stationary phase onset time and
384 final density, despite lacking genes associated with lysogeny. As with our study, the authors observed
385 that this only occurred in liquid culture, and isolation of the virus required numerous rounds of
386 enrichment. They postulated that the virus may cause a successful infection in only a subset of host
387 cells, with the remainder exhibiting alternative interactions such as pseudolysogeny or dormancy
388 [31]. The prevalence of similar infection dynamics in the phages isolated in this study offer two
389 intriguing possibilities: (i) Many of the viruses isolated in this study are either not fully lytic, but fall
390 somewhere on the continuum of persistence [65], controlled by genes currently lacking a known
391 function, with lysogeny (and associated superinfection immunity) favoured at high cell density, in
392 support of the Piggyback-the-Winner hypothesis [66, 67]; (ii) the steady-state of host and virus
393 densities observed here are an indicator of host phenotypic bistability in these streamlined
394 heterotrophic taxa. Viral propagation occurring in only a subset of cells could explain the requirement
395 of multiple rounds of enrichment before sufficient viral load is reached to be able to observe lytic
396 infection on the host population. Either strategy, or a combination of both, would provide an
397 ecological advantage of long-term stable coexistence between viruses and hosts and offer an
398 explanation of the paradox of stable high abundances of both predator and prey across global oceans
399 [12, 39, 68, 69]. Infection in a subset of the population could also explain the low lytic activity
400 observed in pelagiphages *in situ*, despite high host densities [70]; and the small dynamic range and
401 decoupled abundances of SAR11 and virioplankton in the Sargasso Sea [71]. Limited lysis of
402 subpopulations of hosts such as SAR11 and OM43 that specialise in the uptake of labile carbon

403 enriched through viral predation [72, 73] would facilitate efficient intra-population carbon recycling
404 and explain the limited influence of SAR11 and associated viral abundances on carbon export to the
405 deep ocean [5]. We propose the moniker the 'Soylent Green Hypothesis' for this mechanism, after
406 the 1973 cult film in which the dead are recycled into food for the living. Further investigation
407 leveraging our new virus-host model will provide greater insight into viral influence on ocean carbon
408 biogeochemistry.
409
410 In conclusion, our method coupled Dilution-to-Extinction cultivation of hosts and associated viruses,
411 resulting in the isolation of three new strains of OM43; a Western English Channel variant of a warm
412 water ecotype of SAR11; the first known methylophages for OM43; the first siphovirus infecting
413 SAR11, as well as eleven other viruses infecting this important marine heterotrophic clade and >100
414 more isolates to be sequenced and explored. The described method represents an efficient and cost-
415 effective strategy to isolate novel virus-host systems for experimental evaluation of co-evolutionary
416 dynamics of important fastidious taxa from marine and other biomes. Coupling these methods to
417 existing advances in host cultivation requires minimal additional effort and will provide valuable
418 representative genomes to improve success rates of assigning putative hosts to metagenomically-
419 derived viral contigs. Broader representation of model systems beyond cyanophages and viruses of
420 copiotrophic, *r*-strategist hosts will reduce bias in developing methods to delineate viral population
421 boundaries [74, 75], increasing the accuracy with which we derive ecological meaning from viral
422 metagenomic data. We therefore hope that this method will enable viruses to be included in the
423 current resurgence of cultivation efforts to better understand the biology and ecology of phages, and
424 the influence of the world's smallest predators on global biogeochemistry.
425

426 ## Methods Summary

A complete description of the materials and methods is provided in the Supplementary Information. Four bacterial strains (*Methylophilales sp*. C6P1, D12P1 and H5P1; *Pelagibacter sp*. H2P3α) were isolated from Western English Channel station L4 seawater samples using Dilution-to-Extinction methods [28]. All four bacteria and two additional SAR11 strains *Pelagibacter bermudensis* HTCC7211 and *Pelagibacter ubique* HTCC1062 were used as bait to isolate phages from six monthly Western English Channel L4 seawater samples (50°15.00N; 4°13.00W). Briefly, water samples were concentrated for viruses using tangential flow filtration and used as viral inoculum (10% v/v) in exponentially growing cultures of host bacteria in artificial seawater medium [76] in 96-well Teflon plates (Radleys, UK). Cells of the resulting lysate were filtered out and the filtrate was used as viral inoculum in another round of isolation. This process was repeated until viral infection could be detected by flow cytometry - comparing cytograms and maximum density of infected cultures against uninfected cultures. Phages were purified by dilution-to-extinction methods (detailed protocol available here: dx.doi.org/10.17504/protocols.io.c36yrd). Phage genomes were sequenced using Illumina 2x150 PE sequencing, assembled and manually annotated as described in [77]. Phylogenetic classification of phages was performed on concatenated shared genes using a combination of Bayesian inference trees, maximum likelihood trees and shared-gene likelihood analyses, depending on the availability of appropriate taxa. ICTV-recognised genera based on shared gene content were assigned with VConTACT2 [19]. The relative abundance of novel phages in the Western English Channel was calculated by competitive read recruitment using FastViromeExplorer v1.3 [59] against an existing virome from the same location [12].

Data availability All reads can be found in the SRA database under BioProject number PRJNA625644 as BioSamples SAMN14604128-SAMN14604140. Annotated phage genomes are deposited as GenBank submissions under accession numbers MT375519- MT375531.

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Conflict of interests The authors declare no conflict of interest.

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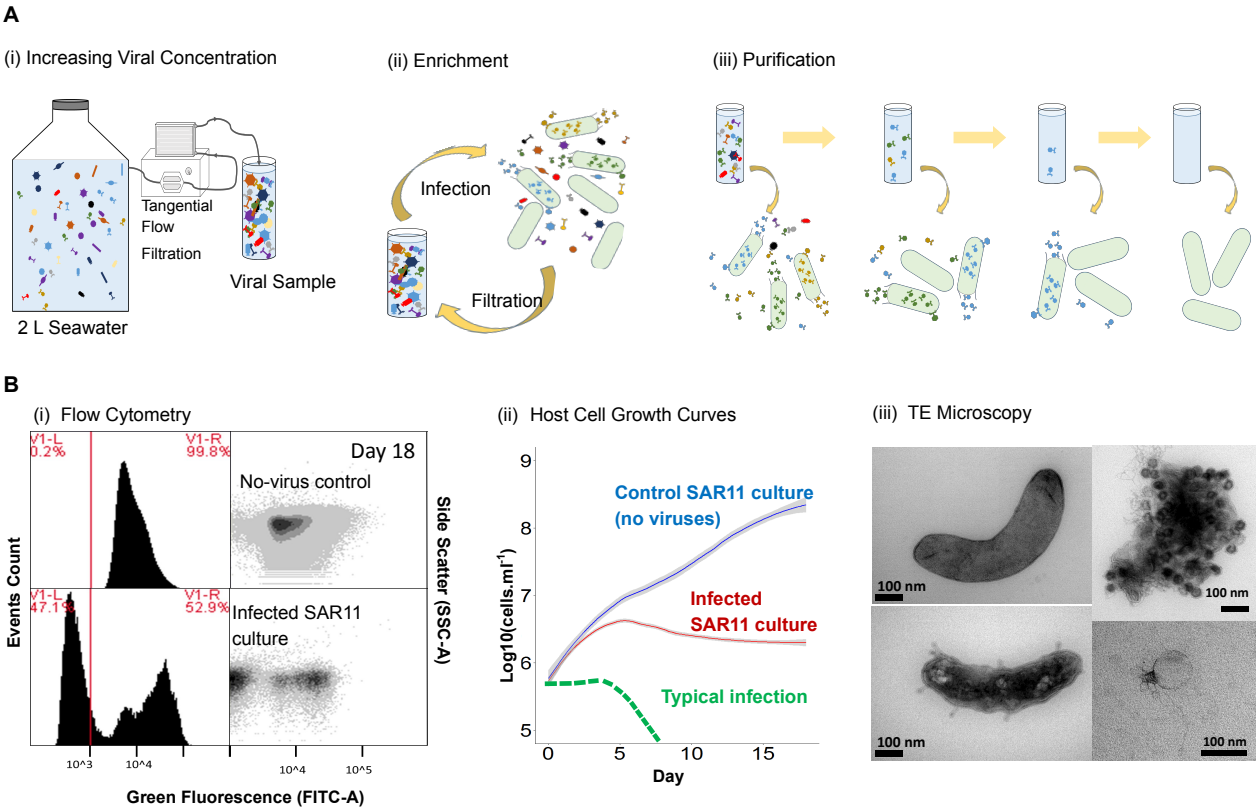


Fig. 1 Workflow for high-throughput isolation **(A)** i. Increasing concentration of viruses in water samples by TFF; ii. Initial infection of host cultures to enrich the sample for specific viruses; iii. Purification of viral isolates through 3 rounds of Dilution-to-Extinction; **(B)** Initial screen of viral infections using i. Flow cytometry, by comparing populations of no-virus controls and infected cultures; ii. Comparing growth curves of no-virus control culture (HTCC1062) against infected SAR11 cultures; iii. Confirming the presence of viruses in infected SAR11 cultures using TE microscopy: top left: HTCC1062 no-virus control, bottom left: infected HTCC1062, top right: aggregated cellular debris and viruses, bottom right: virus found in infected HTCC1062 culture.

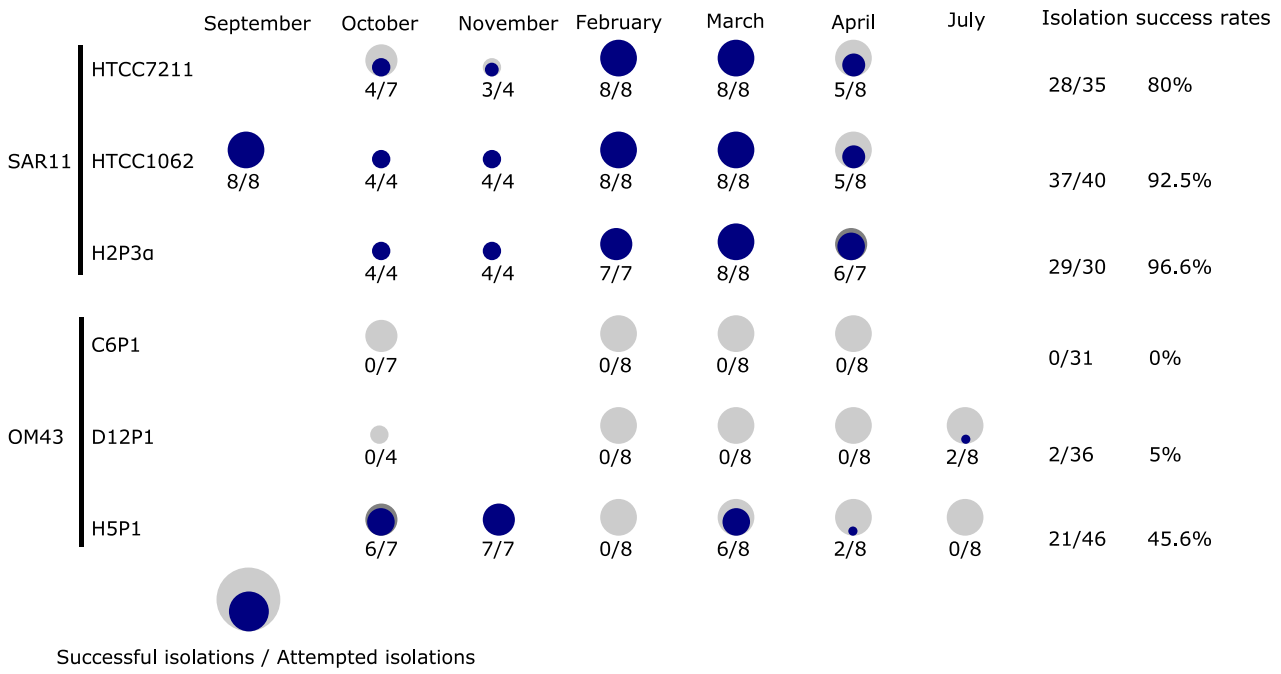
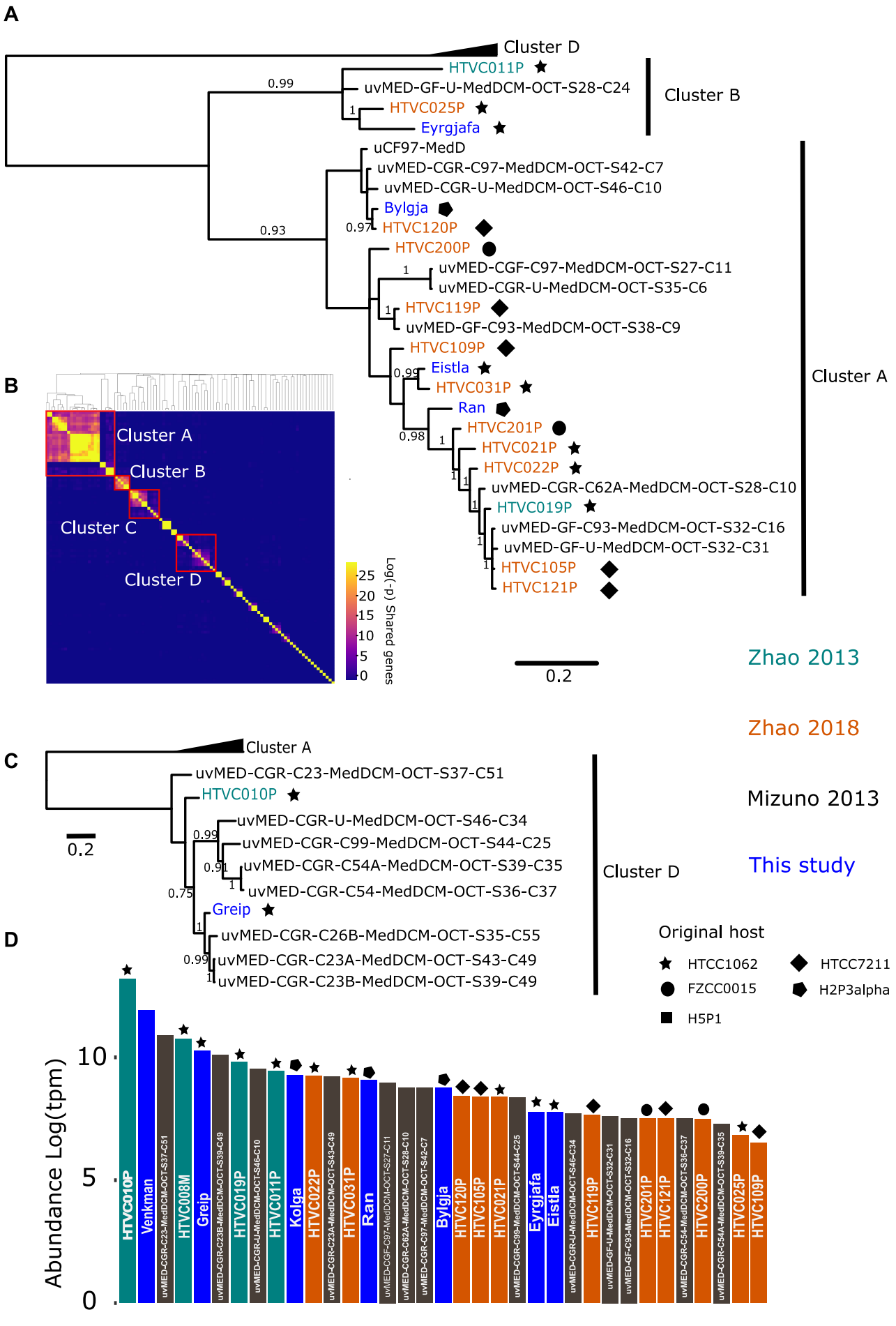
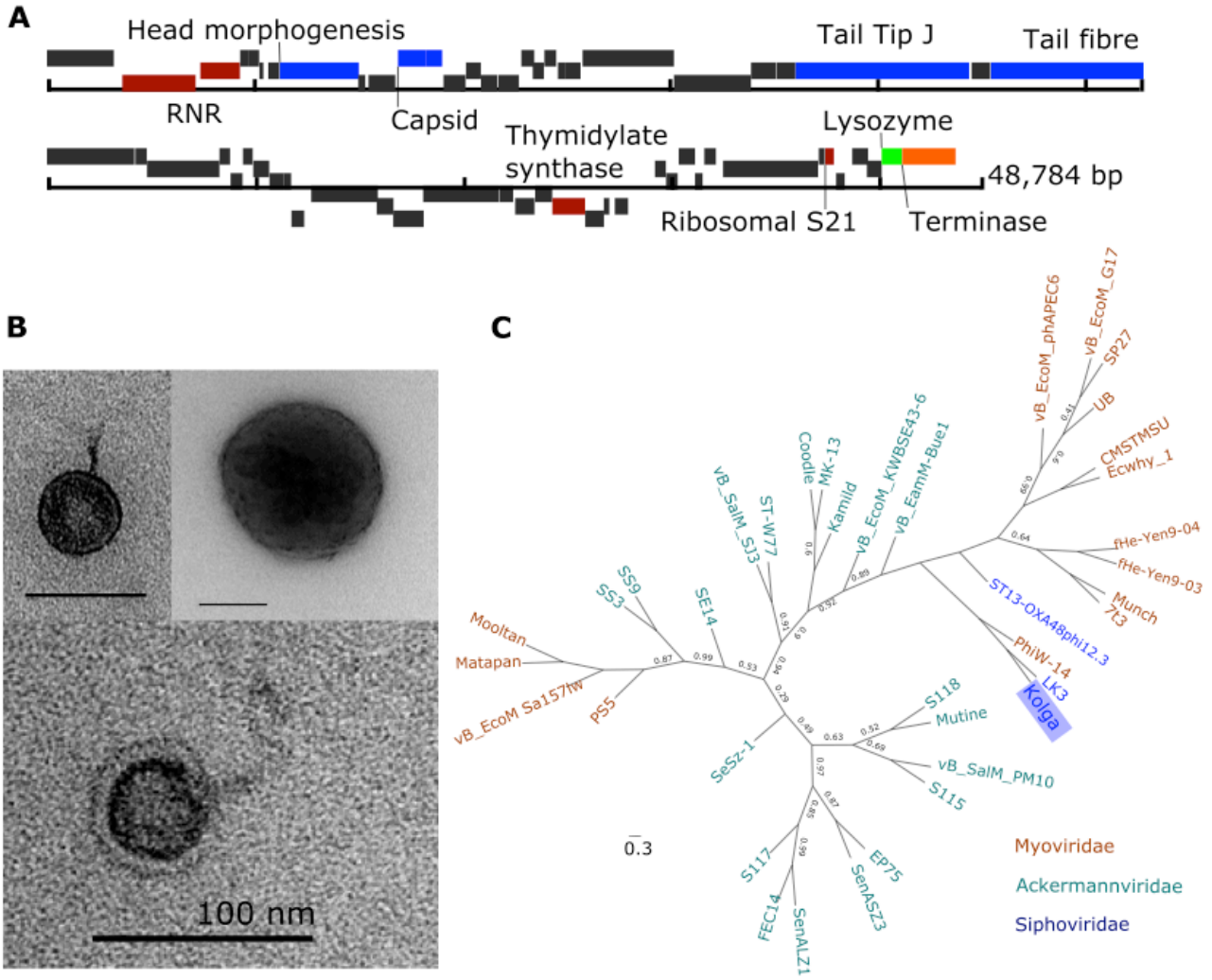


Fig. 2 Summary of success rates for each bacterial host (three SAR11 and three OM43) and water sample combination used for infections; the numerator indicates successful isolation of a virus from that water sample (i.e. successful passage through three rounds of Dilution-to-Extinction), denominator shows the isolation attempts made for a host-water sample combination (i.e. how many cultures were treated with viral samples after multiple rounds of enrichment).



666 Fig. 3 Phylogeny and abundance of the first sequenced pelagiphages from the Western English
667 Channel **(A)** Hypergeometric testing on viral genomes by shared protein content identified four viral
668 clusters and numerous singletons; **(B)** Bayesian inference phylogenetic tree of pelagiphage major
669 capsid proteins from cluster A and B (most diverse pelagiphage cluster, includes most known
670 pelagiphages isolated previously [39, 46] as well as derived from metagenomes [37]), outgroup
671 Cluster D; **(C)** Bayesian inference phylogenetic tree of pelagiphage major capsid proteins from
672 Cluster D (containing the most abundant pelagiphages, including HTVC010P) with Cluster A as
673 outgroup; **(D)** Metagenomic recruitment (reads recruited per million reads (RPM)) of isolate and
674 metagenomically-derived viral genomes against a virome from the Western English Channel [12].
675



676

677 Fig. 4 The first reported siphovirus *Kolga* infecting SAR11 and isolated on novel host H2P3α ; **(A)**
678 gene map of the 48,784 bp genome, which contains 80% hypothetical genes without known function;
679 **(B)** TEMs of *Kolga* (top left and bottom) and a H2P3α host cell infected with *Kolga* (top right); **(C)**
680 Bayesian inference phylogenetic tree of concatenated genes (Pyrophosphohydrolase, thymidylate
681 synthase, two ribonucleotide reductase subunits), all branch support values of 1 have been omitted
682 for clarity

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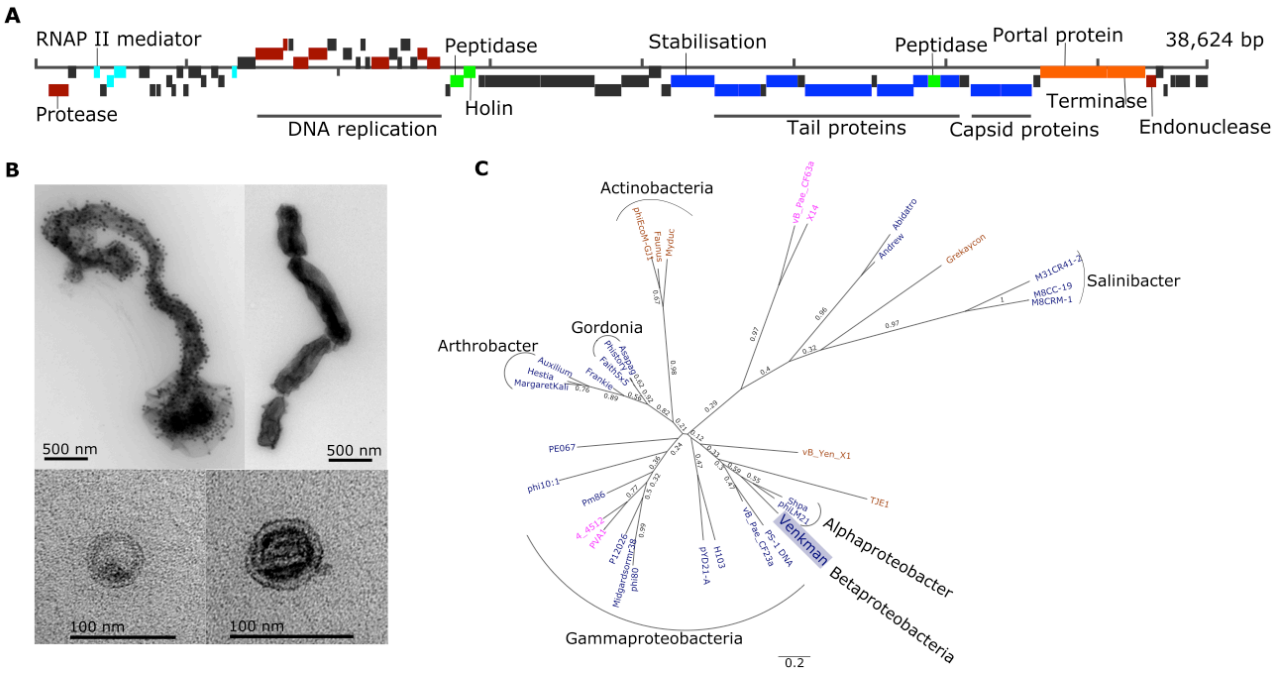

Fig. 5 Evidence for the first reported virus EXEVC282S *Venkman* infecting a member of the OM43 clade (H5P1); **(A)** Gene map displaying protein coding genes. Structural genes are shown in blue, DNA replication genes in red, lysis related genes in green, transcription genes in turquoise, packaging genes in orange, hypothetical genes are grey; **(B)** TEM images of: infected and chaining H5P1 cells (top left), uninfected H5P1 chaining cells (top right), *Venkman* viral particles (bottom left and right); **(C)** Maximum likelihood tree (500 bootstraps) of concatenated viral TerL and exonuclease genes, host families of the phages are indicated on the figure.

694

695 Table 1. Host infectivity of viral populations isolated and sequenced in this study. H2P3 and

696 HTCC7211 are warm-water ecotypes of *Pelagibacter spp*. subclade 1a; HTCC1062 is a cold-water

697 ecotype of *Pelagibacter spp.* Phage *Bylgja* is the only virus known to infect both ecotypes.

Phage	H2P3	HTVC1062	HTVC7211
Eistla		+	
Eyrgjafa		+	
Greip		+	
Ran	+		+
Kolga	+		+
Bylgja	+	+	+

698

699

700 Table 2. Summary of phages isolated and sequenced in this study.

Virus	Host	Viral Population Representative	Hypergeometric cluster	Genome	Length (bp)	G+C%	Taxon	Accession number	Simplified phonetic spelling	Meaning and origin of the names
Eistla	HTCC1062	Eistla	Cluster A	Circular	39638	32.7	Podoviridae	MT375521	aisːtlaː	"stormy one", Giantess in the poetic Edda
Eyrgjafa	HTCC1062	Eyrgjafa	Cluster B	Circular	38005	32.6	Podoviridae	MT375523	eːɪrgjaːfaː	"scar donor", Giantess in the poetic Edda
Gjalp	HTCC1062	Eyrgjafa	Cluster B	Circular	37857	32.5	Podoviridae	MT375524	gjaːlp	"Roaring one", Giantess in the poetic Edda
Greip	HTCC1062	Greip	Cluster D	Circular	34916	31.5	Podoviridae	MT375525	graip	"Grasp", Giantess in the poetic Edda
Ran	H2P3α	Ran	Cluster A	Circular	41529	34.1	Podoviridae	MT375530	ran	"plundering", Goddess of the Sea
Aegir	H2P3α	Kolga	Singleton	Fragment	18297	31.1	Siphoviridae	MT375519	æːgir	"Sea", God of the Sea
Jörmungand	H2P3α	Ran	Cluster A	Circular	41529	34.1	Podoviridae	MT375528	jœrmunˌgand	"huge monster", Giant sea serpent
Kólga	H2P3α	Kolga	Singleton	Circular	48659	30.5	Siphoviridae	MT375529	kɔlgaː	"Cool-wave", Daughter of Ran and Aegir
Unn	H2P3α	Bylgja	Cluster A	Circular	41069	33.5	Podoviridae	MT375531	oːn	"Wave", Daughter of Ran and Aegir
Hroenn	H2P3α	Bylgja	Cluster A	Circular	41069	33.5	Podoviridae	MT375527	hrøn	"Wave", Daughter of Ran and Aegir
Bylgja	H2P3α	Bylgja	Cluster A	Circular	41069	33.5	Podoviridae	MT375520	bɪːlgjaː	"Billow"-wave, Daughter of Ran and Aegir
Himinglæva	H2P3α	Bylgja	Cluster A	Circular	41069	33.5	Podoviridae	MT375526	hiːmɪŋglæfa	"transparent-on-top"-wave, Daughter of Ran and Aegir
Venkman	H5P1	Venkman	Singleton	Linear	38624	34.4	Siphoviridae	MT375522	vɛnkmæn	Bill Murray's Character in the Ghostbusters movie

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