Benchmarking protocols for the metagenomic analysis of stream biofilm viromes

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Viruses drive microbial diversity, function and evolution and influence important biogeochemical cycles in aquatic ecosystems. Despite their relevance, we currently lack an understanding of their potential impacts on stream biofilm structure and function. This is surprising given the critical role of biofilms for stream ecosystem processes. Currently, the study of viruses in stream biofilms is hindered by the lack of an optimized protocol for their extraction, concentration and purification. Here, we evaluate a range of methods to separate viral particles from stream biofilms, and to concentrate and purify them prior to DNA extraction and metagenome sequencing. Based on epifluorescence microscopy counts of viral-like particles (VLP) and DNA yields, we optimize a protocol including treatment with tetrasodium pyrophosphate and ultra-sonication to disintegrate biofilms, tangential-flow filtration to extract and concentrate VLP, followed by ultracentrifugation in a sucrose density gradient to isolate VLP from the biofilm slurry. Viromes derived from biofilms sampled from three different streams were dominated by Siphoviridae, Myoviridae and Podoviridae and provide first insights into the viral diversity of stream biofilms. Our protocol optimization provides an important step towards a better understanding of the ecological role of viruses in stream biofilms.

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Abstract

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Here, we evaluate a range of methods to separate viral particles from stream biofilms, and to concentrate and purify them prior to DNA extraction and metagenome sequencing. Based on epifluorescence microscopy counts of viral-like particles (VLP) and DNA yields, we optimize a protocol including treatment with tetrasodium pyrophosphate and ultra-sonication to disintegrate biofilms, tangential-flow filtration to extract and concentrate VLP, followed by

ultracentrifugation in a sucrose density gradient to isolate VLP from the biofilm slurry. Viromes derived from biofilms sampled from three different streams were dominated by Siphoviridae, Myoviridae and Podoviridae and provide first insights into the viral diversity of stream biofilms. Our protocol optimization provides an important step towards a better understanding of the ecological role of viruses in stream biofilms.

Introduction

Viruses are the smallest and most abundant biological entities on Earth, typically outnumbering their prokaryotic and eukaryotic hosts by an order of magnitude (Rohwer, 2003, Sime-Ngando, 2014). Viruses are a large reservoir of genetic diversity (Suttle, 2007, Sullivan *et al.*, 2017) and occur in all habitats (Paez-Espino *et al.*, 2016), including air (Reche *et al.*, 2018, Rosario *et al.*, 2018), soils (Srinivasiah *et al.*, 2008, Williamson *et al.*, 2017), and deep-sea sediments (Danovaro *et al.*, 2008). The study of viral ecology was pioneered in surface marine systems where prokaryotic viruses, also known as bacteriophages, are the main source of prokaryotic mortality (Suttle, 2007, Brum and Sullivan, 2015, Gregory *et al.*, 2019) and impact ecosystem functions such as the cycling of carbon (Guidi *et al.*, 2016). By lysing their hosts, horizontal gene transfer and metabolic reprogramming, bacteriophages play a pivotal role in structuring microbial communities (Skvortsov *et al.*, 2016, Silva *et al.*, 2017, Rossum *et al.*, 2018, Daly *et al.*, 2019), the flow of energy and matter through food webs (Weitz *et al.*, 2015), the cycling of carbon and nutrients (Dell'Anno *et al.*, 2015, Guidi *et al.*, 2016, Emerson *et al.*, 2018) and the evolution of bacteria (Pal *et al.*, 2007, Rodriguez-Valera *et al.*, 2009, Simmons *et al.*, 2019). Many of these strides have only recently been possible with the advent of molecular tools such as metagenomic sequencing (Rosario and Breitbart, 2011, Brum and Sullivan, 2015, Roux *et al.*, 2016, Roux *et al.*, 2017).

Besides the early recognition of the potential of phages to eradicate bacterial biofilms in medical settings (e.g. Chan and Abedon, 2015), little is known about the interactions between biofilms and viruses (Sutherland *et al.*, 2004). While the biofilm matrix may impede the access of viruses to the surface of bacterial cells (Vidakovic *et al.*, 2017), the susceptibility to phage-induced clearance of biofilm has been demonstrated in laboratory experiments (Scanlan and Buckling, 2012). Biofilms may also act as a reservoir for virus amplification and viruses may endure periods of unfavorable environmental conditions within the extracellular matrix (Doolittle *et al.*, 1996, Briandet *et al.*, 2008). Apart from a few examples (Dann *et al.*, 2016, Silva *et al.*, 2017, Rossum *et al.*, 2018), we lack an understanding of the ecological role of viruses in streams and rivers in general (Peduzzi, 2016) and in stream biofilms in particular (Battin *et al.*, 2016). In streams, biofilms colonize the sedimentary surfaces of the streambed, are biodiversity hotspots comprising members from all three domains of life, and fulfill critical ecosystem processes (Battin *et al.*, 2016). It is reasonable therefore to speculate that viruses also control biodiversity and biomass turnover in stream biofilms with potential consequences for ecosystem functioning and biogeochemical cycling. However, the heterogeneous matrix of stream biofilms has precluded the study of viromes in stream biofilms.

The first critical step towards the study of biofilm viruses in streams requires the effective extraction of viral-like particles (VLPs) from the biofilm matrix. The aim of our study was therefore to optimize laboratory methods for VLP extraction, concentration and purification towards metagenomic analyses. Our effort was specifically tailored to maximize the yield of viral DNA while minimizing DNA contamination from bacteria, eukaryotic hosts or the environment. To this end, we compared a suite of sequential protocols to generate viral metagenomes from stream biofilms including tangential flow filtration (TFF), polyethylene

glycol (PEG) precipitation, physicochemically induced biofilm breakup, and ultracentrifugation followed by nucleic acid extraction. Several of these protocols have been described previously (Vega Thurber et al., 2009, Danovaro and Middelboe, 2010, Hurwitz et al., 2013, Trubl et al., 2016), but their rigorous testing for virome generation from stream biofilms is lacking at present. We benchmark the efficiency of the different protocols using epifluorescence microscopy counts of VLP and DNA yields, and provide first results of the virome structure from biofilms in three streams. We provide a step-by-step version of the optimized protocol at protocols.io, which allows for community participation and continuous protocol development.

Materials & Methods

Sampling

We sampled benthic biofilms from three streams (Switzerland) draining catchments differing in altitude and land use (Table. 1). The Vallon de Nant (VDN) catchment is pristine with vegetation dominated by alpine forests and meadows. The Veveyse (VEV) catchment is characterized by mixed deciduous forests, but also agricultural and urban land use. The Senoge (SNG) catchment is clearly impacted by agricultural land use. During winter, benthic biofilm were randomly collected from stones (5 to 15 cm in diameter) using sterile brushes and Milli-Q water. Slurries were transported on ice to the laboratory pending further processing.

Biofilm properties

For bacterial cell counting, samples were fixed with 3.7% formaldehyde (final concentration) and stored at 4°C. Bacterial cells were disintegrated from the biofilm matrix using 0.25 mM tetrasodium pyrophosphate in combination with rigorous shaking (1 h) and sonication (Bransonic Sonifier 450, Branson) on ice (1 min). Cells were stained using Syto13 and counted on a flow

97 cytometer (NovoCyte, ACEA Biosciences); this protocol worked fine for us previously (e.g.,
98 Besemer *et al.*, 2009). Chlorophyll *a* content was determined spectrophotometrically after over-
99 night extraction in 90% EtOH (Lorenzen, 1967). Extracellular polymeric substances (EPS) were
100 extracted from biofilm slurries using 50 mM EDTA and shaking (1 h) (Battin *et al.*, 2003).
101 Carbohydrates were precipitated in 70% EtOH (-20°C, 48 h) and measured
102 spectrophotometrically as glucose equivalents (DuBois *et al.*, 1956). Proteins were determined
103 according to Lowry (Lowry *et al.*, 1951).
104
105 **Transmission electron microscopy (TEM)**
106 A first confirmation of the presence of VLP in biofilms was obtained from TEM. For this, 5 µL
107 of unprocessed sample was adsorbed to a glow-discharged carbon-coated copper grid (Canemco
108 & Marivac, Canada), washed with deionized water, and stained with 5 µL of 2% uranyl acetate.
109 TEM observations were made on an F20 electron microscope (ThermoFisher, Hillsboro, USA)
110 operated at 200 kV and equipped with a 4098 x 4098 pixel camera (CETA, ThermoFisher).
111 Magnification ranged from 10'000 to 29'000 x, using a defocus range of -1.5 to -2.5 µm.
112
113 **Protocols for the extraction of VLP**
114 We explored a variety of protocols for the concentration, extraction and purification of stream
115 biofilm VLP (Fig. 1). The concentration step is required to obtain sufficient genetic material for
116 nucleic acid extraction. Extraction aims at the liberation of VLP from the biofilm matrix, while
117 purification aims at reducing the amount of contaminant nucleic acids and cellular debris. We
118 tested all possible combinations of protocols to identify the most promising laboratory pipeline
119 for the preparation of viral metagenomes.

First, we homogenized the biofilm slurries by manual shaking and split samples into aliquots (1 L). Aliquots were centrifuged at 100 g (15 min) (5810R, Eppendorf, Hamburg, Germany) to remove sediment particles and larger organisms contained within the biofilm slurry. We recovered the supernatant for downstream analyses.

Concentration

We evaluated tangential flow filtration (TFF) and polyethylene glycol (PEG) precipitation to concentrate the viral size fraction. A medium-scale TFF system equipped with a 100 kDa tangential flow filter (GE Healthcare, USA) was used (Vega Thurber *et al.*, 2009). Viruses were collected in the retentate, whereas water and particles smaller than the pore size were discarded. For each aliquot, the initial volume was reduced to less than 50 mL.

PEG precipitation was performed as described previously (Bibby and Peccia, 2013). Aliquot biofilm samples were supplemented with 10% w/v PEG 8000 (Sigma-Aldrich, Germany) and 2.5 M NaCl (Sigma-Aldrich). The supernatant was then agitated by inverting the tubes three times and stored overnight (4°C), followed by centrifugation for 30 minutes at 9432 g at 4°C (Sorvall RC-5C centrifuge, HS-4 rotor, ThermoFisher). Supernatants were carefully removed and the resulting pellets were eluted with 50 mL sterile H_2O.

Extraction

We tested three different physicochemical treatments (chloroform, tetrasodium pyrophosphate in combination with sonication and dithiothreitol (DTT)) to dislodge viruses from the biofilm matrix. The order - first volume reduction, then chemical detachment - was chosen because the chemicals used for extraction of VLPs may damage TFF filters.

To one subset of the samples, we added 0.2 volumes of chloroform and mixed by inversion. Then, these samples were incubated at 4°C for 30 minutes, vortexed every 5 minutes, and centrifuged at 3234 g for 15 minutes at 4° C (5810R, Eppendorf, Germany) to recover the supernatant (Marhaver *et al.*, 2008, Vega Thurber *et al.*, 2008).

Following the recommendation of Danovaro and Middelboe (2010), we used sonication in combination with tetrasodium phosphate to separate viruses from biofilms. For this, we added 5 mM of tetrasodium pyrophosphate (final concentration) to another subset of concentrated biofilm samples and incubated them in the dark (15 minutes). Then, all samples were sonicated (frequency: 40 KHz; Bransonic Sonifier 450, Branson) three times for 1 minute with 30-second intervals during which the samples were manually shaken. To prevent heating, samples were put on ice during sonication. Finally, one subset of the samples were treated with 6.5 mM DTT and incubated for 1 hour at 37°C. At the end of the incubation period, samples were chilled on ice.

Biofilm samples without chemical treatment served as controls. After each treatment, these samples were first centrifuged (4°C) at 3'234 g for 15 minutes (5810R, Eppendorf) and the supernatant was sequentially filtered through 0.8 µm and 0.45 µm filters (Whatman, GE Healthcare, USA) to remove debris and cells.

Purification

To eliminate contaminating eukaryotic, prokaryotic and extracellular nucleic acids, DNase I at a final concentration of 2.5U/µL (Life Technologies, Germany) and Tris buffer (100 mM Tris pH 7.5, 5 mM $CaCl_2$ and 25 mM $MgCl_2$) were added to the clarified supernatant and incubated at 37°C (2 h). To confirm the removal of contaminant DNA, polymerase chain reaction (PCR) targeting the 16S rRNA gene was performed using the universal primer pair 341f (5′-

CCTACGGGNGGCWGCAG-3′) and 785r (5′-GACTACHVGGGTATCTAAKCC-3′). PCR amplification was performed with a Biometra Thermal Cycler using Taq ALLin polymerase (Axon Lab) according to the manufacturer's instructions with an initial enzyme activation step (95°C for 2 minutes) followed by 30 cycles of denaturation (95°C for 30 seconds) and hybridization (72°C for 30 seconds) and a final elongation (72°C for 10 minutes). PCR products were visualized under UV light after migration on an agarose gel stained using GelRed (Biotium, USA).

For final purification of the viral DNA fraction, two protocols of density gradient ultracentrifugation were assessed. A subset of sample was deposited on top of a CsCl gradient composed of layers of 1 mL of 1.7, 1.5, 1.35 and 1.2 g/mL CsCl, and centrifuged at 106’800 g (Optima XPN-80 Ultracentrifuge, 32 SWi, Beckman Coulter, USA) for 2 h (4°C). The viral fraction was harvested from the interface between the 1.5 and 1.7 g/mL CsCl layers using an 18G needle. Similarly, sucrose gradient ultracentrifugation was assessed for final purification of the viral fraction. The sucrose gradient consisted of 3 mL of 0.2-µm filtered 66% sucrose and 7 mL of 0.2-µm filtered 30% sucrose. The viral fraction was harvested from the interface between both layers using an 18G needle.

Enumerating VLPs using epifluorescence microscopy

To assess the relative viral extraction efficiency of the various protocols, we counted VLP under an epifluorescence microscopy (AxioImager Z2, Zeiss, Germany) as described by Patel et al. (2007). Because of high background noise owing to the biofilm matrix constituents, samples could only be counted after the purification steps. Subsamples were fixed with formaldehyde, stained with SYBR gold (Molecular Probes, ThermoScientific, USA) and incubated at room

189 temperature in the dark (30 minutes). After incubation, each sample was filtered onto a 0.02 µm
190 pore size membrane filter (Anodisc, Whatman). The filters were mounted on glass slides with a
191 drop of VECTASHIELD antifade mounting medium (Vector Laboratories, Burlingame, USA).
192 VLP were visualized using blue light (488 nm) excitation and green (512 nm) emission. For each
193 sample, 15 to 20 randomly selected images were acquired with a camera (Axiocam 506 mono,
194 Zeiss) mounted onto the microscope. VLPs were discriminated from bacteria by size (0.015 to
195 0.2 µm) and enumerated using a custom script in Fiji (Schindelin *et al.*, 2012).
196
197 **Nucleic acid extraction**
198 To further assess the efficiency of the tested protocols to obtain viral nucleic acids for
199 metagenome sequencing, we extracted DNA and quantified its concentration. Following
200 Sambrook et al. (1989), purified viral samples were suspended in 0.1 volume of TE buffer, 0.01
201 volume of 0.5 M EDTA (pH 8) and 1 volume of formamide, and incubated at room temperature
202 (30 minutes). Then, two volumes of 100% EtOH were added and incubated 30 minutes (4°C).
203 Samples were centrifuged at 17'000 g (4°C, 20 minutes) and pellets washed twice with 70% cold
204 EtOH. Pellets were air-dried and resuspended in 567 µL of TE buffer (10 mM Tris and 1 mM
205 EDTA (pH 8.0)). Thirty µL of warm 10% SDS and 3 µL of proteinase K (20 mg/mL) were
206 added and samples were incubated for 1 h at 37°C. Next, 100 µL of 5M NaCl and 80 µL of
207 warm CTAB were added and incubated for 10 minutes (65°C). DNA was extracted in a series of
208 chloroform, phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform treatments, with
209 centrifugation at 16'000 g (10 minutes) for phase separation. Finally, DNA was precipitated
210 overnight in isopropanol (−20°C). DNA was concentrated by centrifugation at 16'000 g (4°C)
211 for 20 min, and the pellet washed twice with cold 70% EtOH, air-dried, resuspended in 50 µL

nuclease-free H_2O and stored (−20°C). DNA concentration was measured using Qubit and the dsDNA high-sensitivity kit according to the manufacturer's instructions (Life Technologies, Carlsbad, USA).

Library construction and sequencing

Based on the epifluorescence microscopy counts of VLP and the DNA concentration from purified biofilm samples, we selected samples processed with the best performing pipeline for metagenome sequencing. For sequencing library construction, DNA was sheared with an S2 focused ultrasonicator (Covaris, Woburn, USA) to achieve a target size of DNA fragments of around 350 bp. We opted for the ACCEL-NGS® 1S PLUS DNA library kit (Swift Biosciences, USA) which allows low quantities of both single- and double-stranded DNA as input (Roux *et al.*, 2016). Library construction and multiplexing was performed following the manufacturer's instructions for DNA inputs (<1 ng/µL) and 20 cycles of indexing PCR. Paired-end sequencing (2 x 300 bp) was performed on a MiSeq System (Illumina, San Diego, USA) at the Lausanne Genomic Technologies Facilities. Raw sequences have been submitted to the European Nucleotide Archive under accession number PRJEB33548.

Bioinformatic analyses

The number of reads and GC content of each sample before and after quality control were calculated using a custom python script. For virome classification, we followed the recommendations to assemble viral contiguous sequences (contigs) according to Roux et al. (2019). First, BBDuk (v35.79) was used to remove Illumina adapters, filtering and trimming (trimq=12). Next, reads with >93% similarity to a human reference genome were discarded

(using BBmap). The ACCEL-NGS® 1S Plus library preparation kit includes a low complexity adaptase tail, which was clipped (10 bases) according to the manufacturer's instructions. We then used the error correction capability of Tadpole (v. 37.76) to correct for sequencing errors (mode=correct ecc=t prefilter=2). Prior to assembly, we de-duplicated our datasets using clumpify (v37.76) with parameters set such that identical reads were identified and only one copy was retained (dedupe subs=0). Finally, we used the SPAdes assembler (v3.13.0, Bankevich *et al.*, 2012) in single-cell (--sc) mode, with error correction disabled (--only-assembler) and kmers set to 21, 33, 55, 77, 99, 127 to assemble contigs. We co-assembled the paired-end reads from both sequencing runs for each sample individually. To obtain an overview of the potential contaminant sequences (e.g., human, bacterial and phiX), we uploaded the quality-trimmed and de-replicated reads (forward orientation only) to the web interface of taxonomer (Flygare *et al.*, 2016). Taxonomer is an ultrafast taxonomy assigner, which assigns and classifies reads to human, bacterial, viral, phage, fungal, phix, ambiguous (i.e., reads fit to more than one bin) and "unknown" bins. For identification and classification of viral contigs, we used MetaPhinder (v2.1, Jurtz *et al.*, 2016) through the web interface hosted at the Center for Genomic Epidemiology at the Danish Technical University (DTU). Additionally, we queried the Viral_rep and Phage_F domains of the PFAM database using hmmsearch (HMMER v3, Eddy, 2011) to identify ssDNA contigs (Trubl *et al.*, 2019).

Results

Biofilm properties

Bacterial abundance ranged between 4.1×10^{11} cells m^{-2} at the lowest stream (SNG) and 2.3×10^{9} cells m^{-2} at the uppermost stream (VDN; Table 1). This pattern was mirrored by an increase in chlorophyll *a* content, and the protein and carbohydrate concentrations in EPS that were

higher in the lowland than the high-altitude stream (Table 1). Despite these differences in biofilm properties, no differences in extraction efficiency among the different protocols were detected. In fact, the average number of VLPs extracted by the various protocols were strongly correlated among the different samples (pairwise Spearman's r_s ranging between 0.94 and 0.98).

Transmission Electron Microscopy (TEM)

Direct electron microscopic observations of raw biofilm samples revealed the presence and morphological diversity of virions, including tailed bacteriophages and lemon-shaped viruses, in biofilms from all three streams (Fig. 2). Polyhedral, spherical and filamentous VLPs were also observed, which may include untailed bacteriophages or viruses infecting eukaryotes.

Virome extraction and purification

In total, we evaluated 16 protocols to concentrate, extract and purify viruses from benthic biofilms from three streams (Fig. 1). Based on the number of VLPs and DNA yields retained at the end of each protocol, we observed significant differences in relative extraction efficiencies among protocols. Across all samples, average VLP counts and DNA yields were correlated (Spearman's r_s = 0.74), suggesting conformity among these two means of evaluation. The combination of TFF for concentration, tetrasodium pyrophosphate and sonication for extraction, and sucrose gradient centrifugation for purification resulted in the highest VLP counts in all three samples (two-way ANOVA, $p < 0.01$; Fig. 3). This pipeline also yielded highest DNA concentration (Fig. 4). Protocols involving PEG precipitation generally resulted in a lower recovery of VLPs (on average only 13.6 % compared to the best performing pipeline) and DNA yields below detection limit. This may be attributable to the formation of a visible, viscous layer

upon addition of PEG to the biofilm samples. The dissociation of VLPs from the biofilm matrix was most effective using tetrasodium pyrophosphate and sonication (two-way ANOVA, $p<0.01$). Protocols based on TFF and using DTT or chloroform extracted on average 25.0% and 33.2% less VLPs than protocols using TFF followed by tetrasodium pyrophosphate treatment and sonication (Fig. 3). Samples without any physicochemical treatment to extract VLP from biofilms yielded on average 54.8% less VLPs than the tetrasodium pyrophosphate and sonication treatment. To further purify viruses, two discontinuous gradient formulations with sucrose and CsCl were tested. On average, 1.9 times more VLPs were retained by ultracentrifugation using the sucrose gradient than using the CsCl gradient in samples concentrated using TFF and treated with tetrasodium pyrophosphate and sonication (paired-T-Test, $p<0.01$). This is also reflected in DNA yields, which reached 1.22 ng μL^{-1} in VDN, 1.31 ng μL^{-1} in VEV and 18.7 ng μL^{-1} in SNG using TFF, pyrophosphate and sonication followed by sucrose gradient centrifugation. DNA yields were on average 3 times higher using ultracentrifugation in the sucrose compared to the CsCl gradient (paired T-Test, $p=0.03$) and on average 1.5, 1.7 and 1.9 times higher using tetrasodium pyrophosphate and sonication compared to DTT, no physico-chemical detachment and chloroform, respectively.

Given that stream biofilms contain abundant prokaryotic, eukaryotic and extracellular DNA, it is crucial to verify the absence of DNA potentially contaminating the samples. Negative PCR results from samples treated with DNase I confirmed the absence of DNA contamination from prokaryotic cells.

Stream biofilm viromes

From the two sequencing runs we obtained 24‘388‘096, 22’459’218 and 23’804’190 paired-end reads from SNG, VEV and VDN, respectively (Table 2). After quality control, error correction and deduplication, on average 97.5% of the reads remained. Initial screening using taxonomer showed that human contaminant reads accounted for 0.13 to 0.25% of the reads, while bacterial contaminant reads accounted for 1.47 to 8.11% of the reads.

We obtained 3698, 11’323 and 13’591 contigs from de-novo assembly of quality-controlled and deduplicated reads from SNG, VEV and VDN, respectively. The largest contigs were 9493, 46’665 and 54’492 bp in SNG, VEV and VDN, respectively. Hmmsearch against the PFAM databases did not yield ssDNA contigs in the three viromes. Between 726 and 2613 contigs were classified as of viral origin in the three viromes (Fig. 5). In all samples, the majority of contigs were identified as not further classified Siphoviridae (28.3 – 53.3%), followed by Myoviridae (9.6 – 18.0%) and Podoviridae (4.7 – 5.2%). Contigs classified as T4virus, Cp220virus, Kayvirus and P12024virus were common in all three biofilm viromes. However, contigs classified as Twortvirus, Phicbkvirus, Coopervirus and L5virus were only detected in viromes obtained from VEV and VDN but not in SNG.

Discussion

The advent of metagenomic tools has revolutionized the study of the role of viruses in numerous ecosystems (Suttle, 2007, Rosario and Breitbart, 2011, Brum and Sullivan, 2015, Trubl *et al.*, 2019). For biofilms in general, and particularly for biofilms in streams and rivers, however, an optimized protocol for viral metagenomics has been missing. Here, we establish an optimized sample-to-sequence pipeline for the concentration and purification of viruses from stream biofilms. This protocol is publicly available at https://www.protocols.io/view/extraction-and-purification-of-viruses-from-stream-32qgqdw. We used two metrics, the number of VLP retained

328 and the amount of DNA extracted from the samples to evaluate the performance of the different
329 combinations of protocols (Fig. 3, Fig. 4). This approach allowed us to obtain a relative
330 comparison among the tested protocols. However, it does not permit the quantification of
331 extraction efficiencies since it was not possible to enumerate VLPs without prior extraction and
332 purification.
333 The best performing sample-to-sequence pipeline involves TFF for sample concentration,
334 pyrophosphate and sonication for the detachment of viruses from the biofilm matrix and DNase I
335 treatment followed by sucrose gradient ultracentrifugation for purification. This is similar to
336 protocols for other complex samples, such as soils (Trubl *et al.*, 2016) or marine and freshwater
337 sediments (Danovaro and Middelboe, 2010). The suggested combination of protocols was
338 consistently the best performing pipeline for biofilms obtained from three streams differing in
339 trophic state and with different biofilm properties (Table 1).
340 The viromes obtained using the best-performing protocol were dominated by reads of viral or
341 unknown origin (presumably reflecting the lack of viral sequences in public databases), and
342 contaminant sequences (i.e., of bacterial or human origin) contributed only marginally to the
343 viromes. Following an optimized assembly strategy (Roux *et al.*, 2019), the reads assembled into
344 a large number of contigs, however, of small average contig size. Still many of the contigs were
345 classified as of viral origin. Viral community composition was remarkably similar across the
346 three different stream biofilms, with several contigs classified as T4virus, Cp220virus, Kayvirus
347 and P12024virus found in all samples. Despite the use of the ACCEL-NGS® 1S PLUS DNA kit
348 (Roux *et al.*, 2016), ssDNA viruses, which account for <5% of DNA viral communities in other
349 freshwater, marine and soil ecosystems (Roux *et al.*, 2016, Trubl *et al.*, 2019) could not be
350 detected in the viromes from stream biofilms. Strikingly, the virome obtained from the most

eutrophic stream (SNG) resulted in the lowest number of viral contigs and lacked contigs classified as Twortvirus, Phicbkvirus, Coopervirus and L5virus. However, given the low number of samples, we caution against concluding an anthropogenic effect on stream biofilm viral communities.

A range of chemical properties may explain the relative differences in virus extraction efficiency from stream biofilms. PEG precipitation generally failed to concentrate viruses from biofilm slurries, potentially due to the formation of a viscous layer that impaired PEG removal. Previously described methods for isolating viruses involve chloroform (Marhaver *et al.*, 2008, Vega Thurber *et al.*, 2008, Hewson *et al.*, 2012); however, chloroform may denature the lipid envelopes surrounding viral capsids, internal lipid membranes and nucleo-cytoplasmic large DNA viruses (NCLDV), including Phycodnaviridae, which predominantly infect freshwater and marine algae, may be sensitive to chloroform treatment (Feldman and Wang, 1961). Moreover, ssDNA viruses lack a lipid envelope and some tailed bacteriophages may display sensitivity to chloroform. Similarly, DTT is a reducing agent, which breaks disulfide bonds in proteins. This may explain to the reduced recovery of VLPs from samples treated with PEG, chloroform or DTT.

Extracellular DNA is a common component of the biofilm matrix (Flemming and Wingender, 2010), which together with DNA from damaged eukaryotic and prokaryotic cells needs to be removed prior to virome sequencing. We propose a DNase I treatment as an efficient way to digest extracellular DNA from biofilms, followed by sucrose density gradient ultracentrifugation for further purification. This order is important because otherwise DNA from viral particles damaged during ultracentrifugation may be digested by the DNase digestion. Compared to ultracentrifugation in a CsCl gradient, sucrose gradient ultracentrifugation probably maximized

the purification of a wider range of viruses because of the larger gradient of densities recovered with this method. Moreover, CsCl density gradient separation may exclude viral particles as that may be too buoyant (Vega Thurber *et al.*, 2009), or degrade the structure of enveloped viruses (Lawrence and Steward, 2010) and therefore reduce their recovery. A critical step of virome preparation concerns the nucleic acid extraction. We chose a derivation of a standard protocol, which allows the extraction of both ssDNA and dsDNA viruses (Sambrook *et al.*, 1989).

Conclusions

In conclusion, we provide a first protocol for the generation of viromes from stream biofilms. This paves the way for a better understanding of the roles viruses may play in stream ecology. By providing a step-by-step protocol on protocols.io, we hope to further stimulate research on phage diversity in stream biofilms.

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535

Table 1(on next page)

Sample site and biofilm characteristics.

	VDN	VEV	SNG
Coordinates	46°15'13.5"N 7°06'33.9"E	46°30'46.4"N 6°54'43.7"E	46°33'23.9"N 6°28'55.3"E
Altitude (m a.s.l.)	1210	766	498
Bacterial abundance [cells m^{-2}]	2.3×10^{9}	1.8×10^{10}	4.1×10^{11}
Chlorophyll-a [µg cm^{-2}]	0.21	0.22	1.21
EPS proteins [µg cm^{-2}]	0.05	0.04	0.24
EPS carbohydrates [µg cm^{-2}]	below detection	below detection	0.08

1

Table 2(on next page)

Virome dataset statistics

	raw reads		quality trimmed			
	paired-end sequences	**GC content (%)**	**paired-end sequences**	**GC content (%)**	**Contigs**	**Average contig length (bp)**
SNG	24 388 096	40.36	24 172 084	40.16	3698	452
VEV	22 459 218	40.51	22 153 818	40.24	11323	645
VDN	23 804 190	40.68	23 422 408	40.21	13591	676

1

Figure 1(on next page)

Overview of methods for the extraction and purification of viruses from stream biofilms.

First, phages are concentrated using either PEG precipitation or TFF. Different physico-chemical extraction procedures were then evaluated for their efficiency. Prior to DNase I digestion, centrifugation and filtration was used to remove cell debris from all samples. Finally, ultracentrifugation in CsCl or sucrose gradients was used to purify viruses for downstream molecular analyses. Combinations of all protocols were evaluated for the recovery of VLPs and DNA yield.

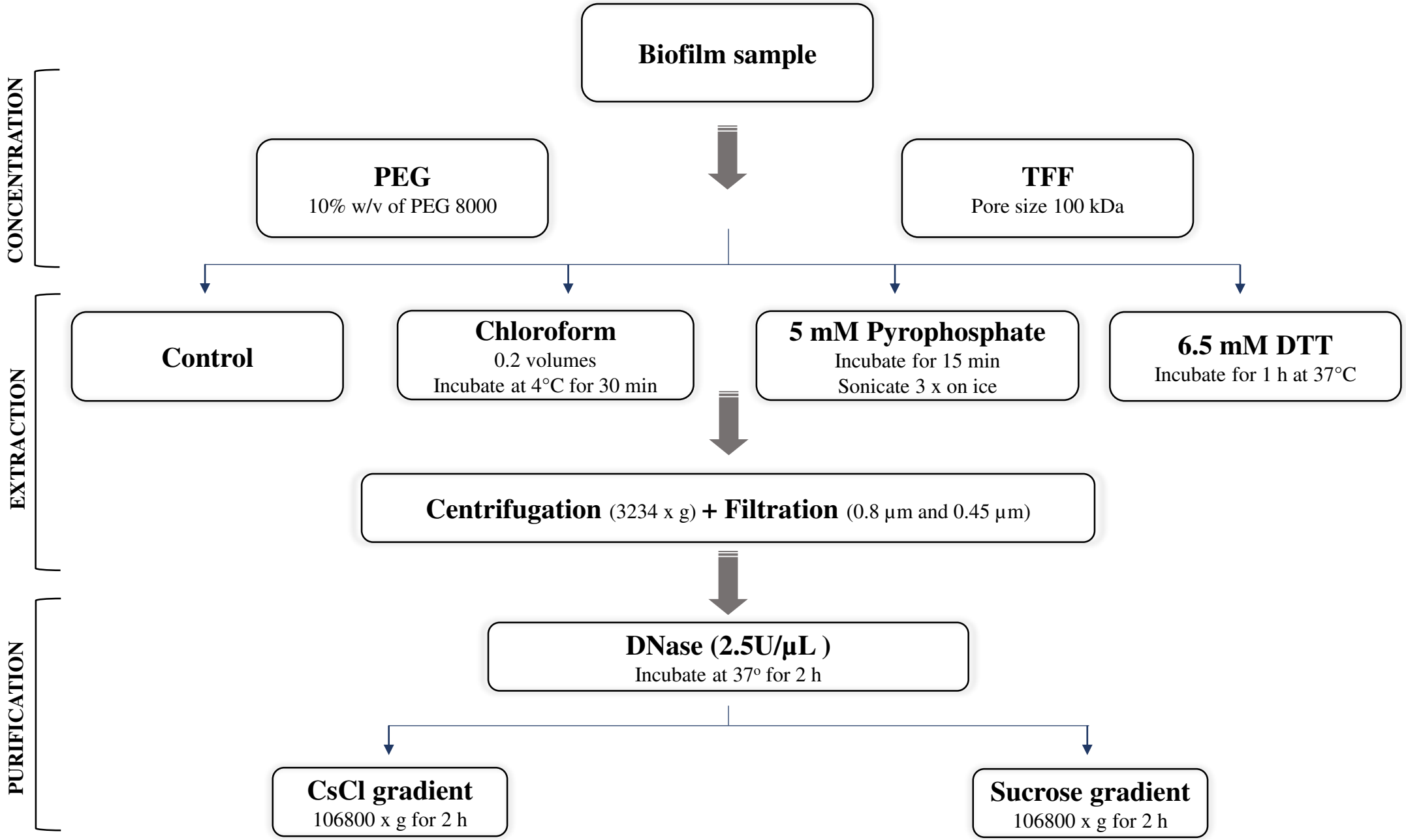

CONCENTRATION
EXTRACTION
PURIFICATION
Biofilm sample
PEG
10% w/v of PEG 8000
TFF
Pore size 100 kDa
Control
Chloroform
0.2 volumes
Incubate at 4°C for 30 min
5 mM Pyrophosphate
Incubate for 15 min
Sonicate 3 x on ice
6.5 mM DTT
Incubate for 1 h at 37°C
Centrifugation (3234 x g) + Filtration (0.8 µm and 0.45 µm)
DNase (2.5U/µL)
Incubate at 37º for 2 h
CsCl gradient
106800 x g for 2 h
Sucrose gradient
106800 x g for 2 h

Figure 2

Electron microscopic evidence of virus-like particles in stream biofilm samples.

A large morphological diversity of VLPs, including tailed bacteriophages, lemon-shaped, polyhedral, spherical and filamentous viruses was observed using TEM.

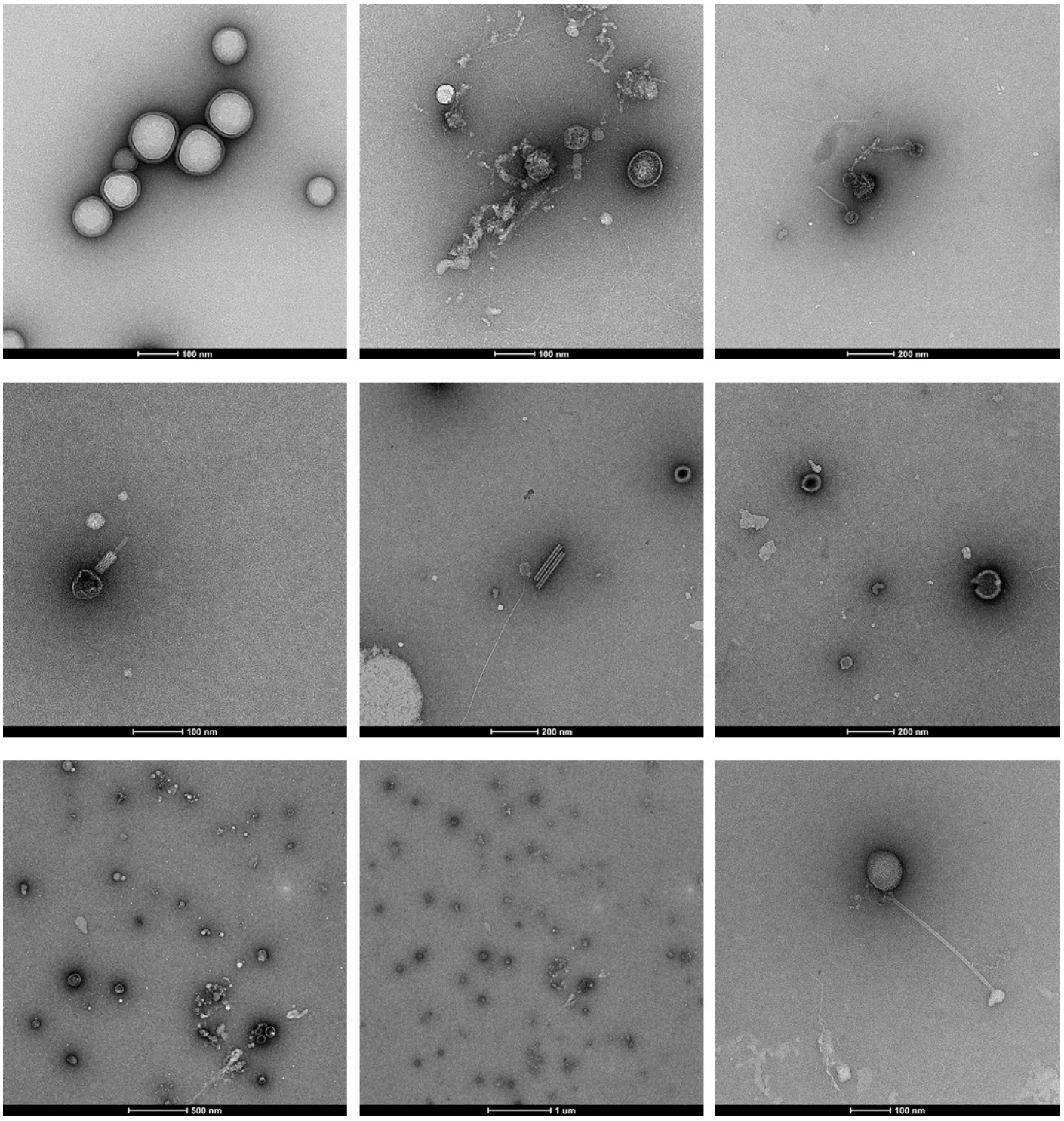

100 nm
100 nm
200 nm
100 nm
200 nm
200 nm
500 nm
1 um
100 nm

Figure 3(on next page)

VLP yields of the different combination of methods for the extraction and purification of viruses from stream biofilms.

The boxes show the number of VLPs in replicated subsamples processed with each protocol, the median is given as a horizontal line, hinges correspond to the first and third quartile while whiskers extend to the largest and smallest values counted. In each of the three streams tested, a protocol based on TFF, tetrasodium pyrophosphate in combination with sonication and ultracentrifugation in sucrose gradient yielded significantly higher VLP counts than any other combination of protocols.

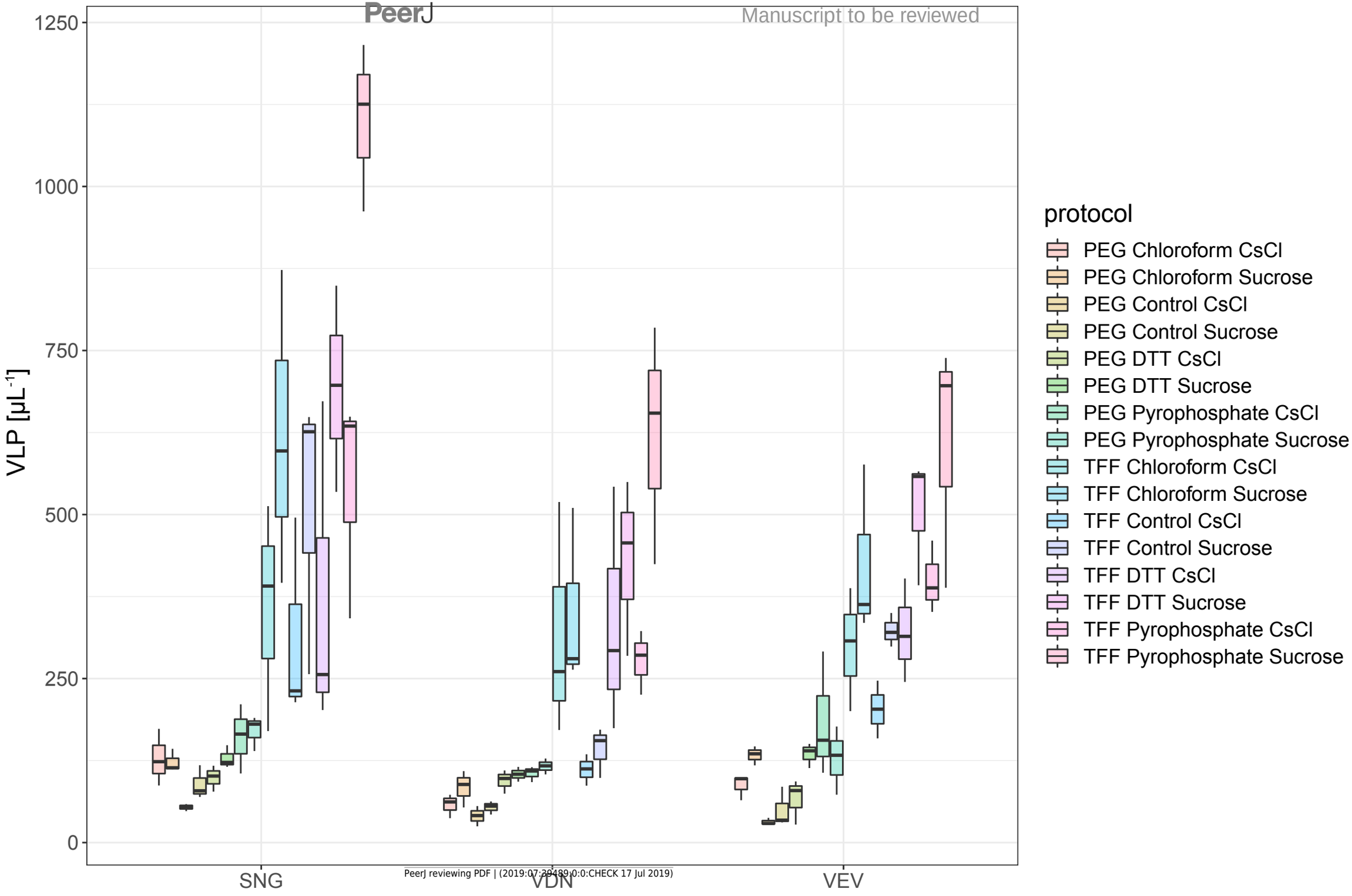


Figure 4(on next page)

DNA yields from samples processed with different protocols.

None of the protocols involving PEG precipitation resulted in detectable DNA yields (not shown). Protocols using TFF and either no treatment (control), chloroform, DTT, tetrasodium pyrophosphate in combination with sonication for extraction and either CsCl or Sucrose gradient ultracentrifugation for purification yielded between 0.5 and 18.7 ng DNA μL^{-1}. Note that although VLP counts were only 1.8 times higher in the best performing protocol in SNG as compared to the other two samples (VDN, VEV), DNA yields in this sample were more than 14 times higher.

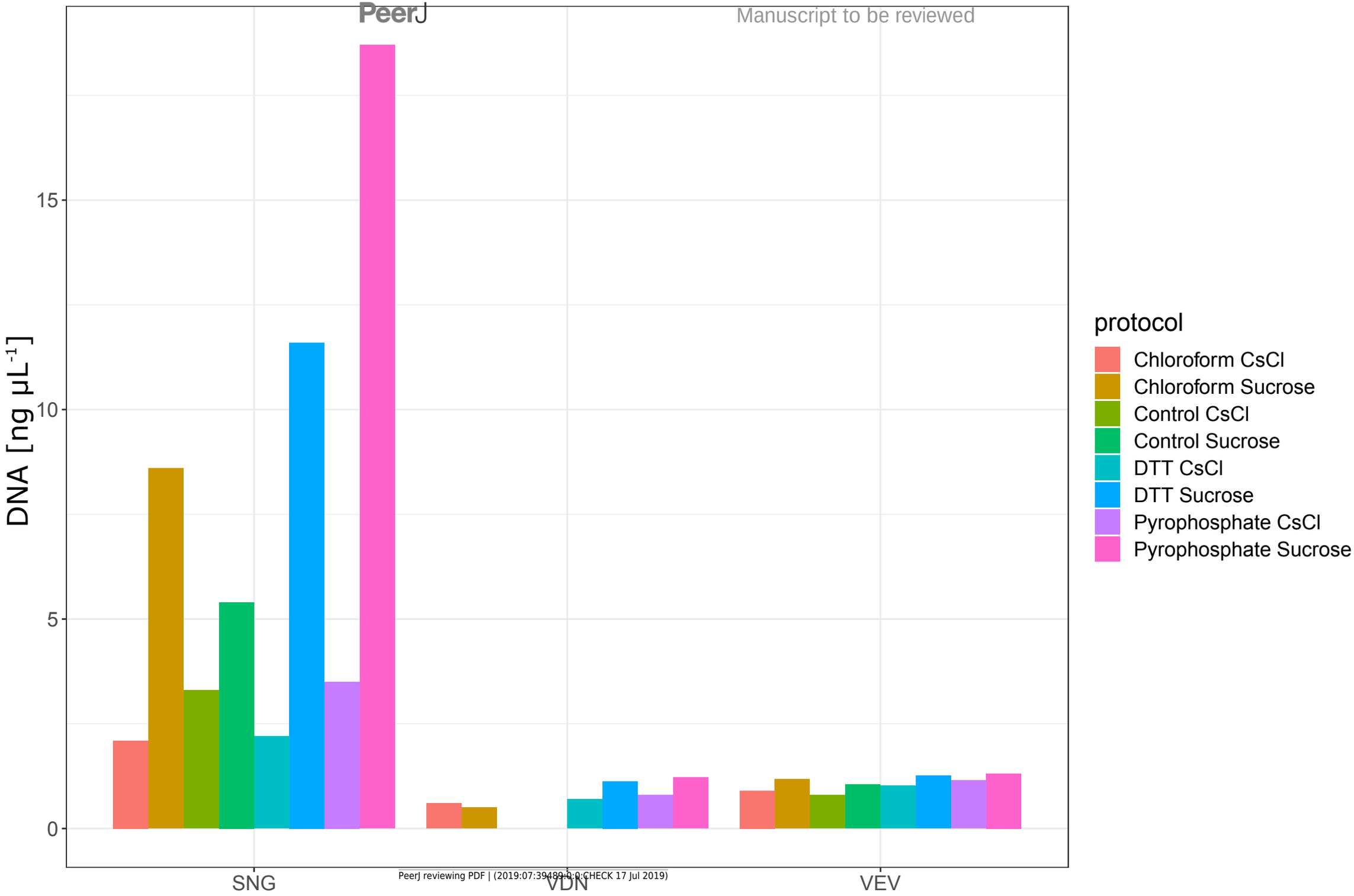
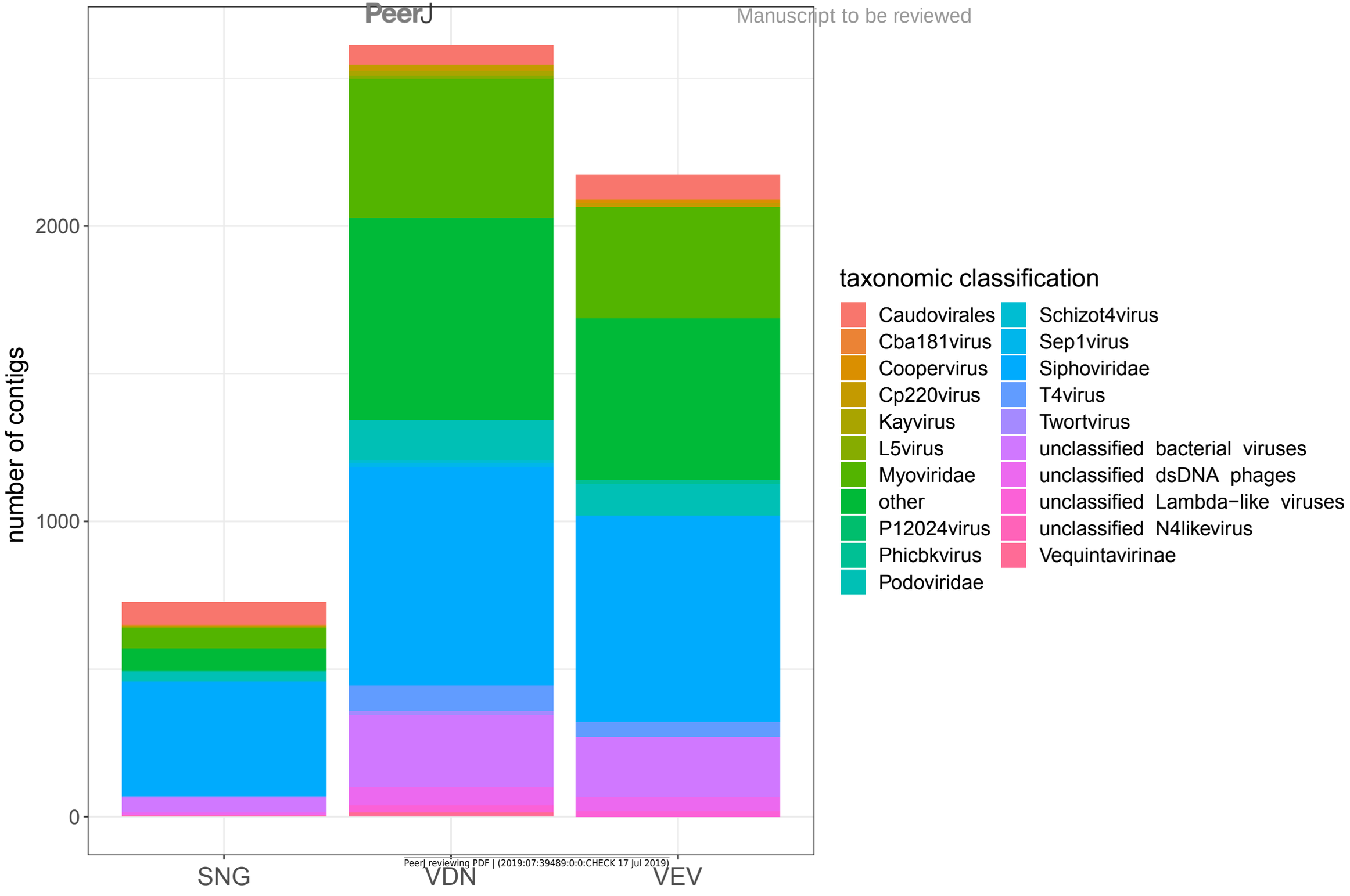

DNA [ng µL-1]
15
10
5
0
SNG
VDN
VEV
protocol
Chloroform CsCl
Chloroform Sucrose
Control CsCl
Control Sucrose
DTT CsCl
DTT Sucrose
Pyrophosphate CsCl
Pyrophosphate Sucrose

Figure 5(on next page)

Viral contig taxonomic composition.

Taxonomic classification after contig assembly showed that stream biofilm viromes were dominated by not further classified members of Siphoviridae and Myoviridae. The relative composition of contigs was similar among the three samples, however, markedly fewer contigs were obtained from SNG than from the other two stream biofilm samples. ssDNA viruses could not be identified among contigs from any of the streams.


number of contigs
2000
1000
0
SNG
VDN
VEV
taxonomic classification
Caudovirales
Cba181virus
Coopervirus
Cp220virus
Kayvirus
L5virus
Myoviridae
other
P12024virus
Phicbkvirus
Podoviridae
Schizot4virus
Sep1virus
Siphoviridae
T4virus
Twortvirus
unclassified bacterial viruses
unclassified dsDNA phages
unclassified Lambda−like viruses
unclassified N4likevirus
Vequintavirinae