Lines 38–41 “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).”

Prokaryotic viruses are not known as bacteriophages. The word Prokaryotic is used to describe bacteria and archaea (which I personally do not like, but the field is split). Bacteriophage is used to describe viruses that infect bacteria only. Now, most of the viruses in the references you list are bacteriophage, therefore you should replace Prokaryote with bacteria.

Additionally, Guidi et al. (2016) does show what you say, but specifically that cyanophage are most correlated with carbon transport from surface waters to the deep ocean. I recommend citing one of Mya Breitbart’s excellent reviews (https://www.nature.com/articles/s41564-018-0166-y)

Please rewrite this sentence with something similar to this “The study of viral ecology was pioneered in surface marine systems where viruses that infect bacteria, also known as bacteriophages, are the main source of bacterial mortality (Suttle, 2007, Brum and Sullivan, 2015, Gregory et al., 2019) and impact ecosystem functions such as the cycling of carbon (Breitbart et al. 2018).”

Lines 74–76: “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.

I think some writing is missing from this sentence. Also, this is the first mention of viromes and providing literature context. Most of the intro should be on this and biofilms in streams.

“We provide a step-by-step version of the optimized protocol at protocols.io, which allows for community participation and continuous protocol development.”

This is great!

Introduction: I think you have done an excellent review of the literature, but only for bulk metagenomes of viruses. Your paper describes doing a VLP separation first and then sequencing which is a virome, but there is no background on virome or cited literature. Please cite some of these and describe why do a virome: Matt Sullivan lab viromes papers (e.g. Trubl et al; Duhaime et al), Mya Breitbart (e.g. Angly et al. 2006). There are many more out there.

Please change Vega Thurber to Thurber for parenthetical citations and in references.

Section “Protocols for the extraction of VLP”

I am confused here, did you do concentration before VLP extraction? You say you took the virus size fraction, but then dislodge from biofilms? This makes no sense, because VLPs in a biofilm would be much larger than the VLP fraction. Please clarify.

Okay…I see in the next section you state why (and the figure 1 is clear), but please have a summary paragraph at the beginning that walks us through this. I am still confused how things were of virus size if in a biofilm.

In Figure 1, please list the density gradients used. CsCl: "1 mL of 1.7, 1.5, 1.35 and 1.2 g/mL" and sucrose: "3 mL of 0.2-µm filtered 66% sucrose and 7 mL of 0.2-µm filtered 30% sucrose"

I am also concerned with the density gradients that were used and collected. The Thurber et al. (2009) paper is cited here and they laid the foundation of viromes. That paper and a slew of following papers show that dsDNA viruses are in CsCl density gradients ~1.4–~1.5 and for ssDNA viruses collect ~1.3. In this paper all the right density gradients were used, but you say you only collected "1.5 and 1.7 g/mL". This is very troubling because you will definitely get some viruses, but miss a large portion of dsDNA viruses and all ssDNA viruses.

Lines 173–174 "For final purification of the viral DNA fraction, two protocols of density gradient ultracentrifugation were assessed."

CsCl and sucrose cushion are used to purify the virus particles and not their DNA. Please rewrite to specify the purification of virions via density separation.

Lines 186–187: "Because of high background noise owing to the biofilm matrix constituents, samples could only be counted after the purification steps."

What about the pyrophosphate? I know it was below 10mM, but was there any background noise? It might be nice to cite that 10mM and greater concenrtrations see a lot of this and lower ones do not.

Lines 235–242: Referring to the read correction and assembly protocol.

The pipeline used here is exactly what was published in Roux et al. 2019, but it is not cited here, only in the discussion.

Lines 250–252 "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)."

I am not following this. How did you use hmmsearch to query Pfam? More writing is needed to clarify. I think you annotated your viral contigs using Pfam database, used these sequences as models for a hmmsearch?

347–350 "Despite the use of the ACCEL-NGS® 1S PLUS DNA kit (Roux *et al.*, 2016), ssDNA viruses, which account for <5% of DNA viral communities in other freshwater, marine and soil

ecosystems (Roux *et al.*, 2016, Trubl *et al.*, 2019) could not be detected in the viromes from stream biofilms."

ssDNA viruses were not detected because you did not collect the correct density gradients. I would state this as an explanation to why they were not present.

The TEM micrographs are great. I would like some discussion of the different morphotypes, because some of them look similar to archaeal viruses.

Figure 4:
Legend has results in it. Please remove these.
The previous box plots looked great, why did you switch to bar charts? Please redo as box plots.
Some of the samples did not yield DNA? Please denote as not detectable.

Figure 5:
The legend is not descriptive of the figure, but rather just results.
I recommend remaking this figure to make it easier for the reader. Transform into a proportional stacked bar chart that is set to 100%. This way the relative abundance of each taxonomic group can be compared across samples and have above the total number of contigs to convey virus richness.
Also, this viruses could easily be clustered into viral populations (see Roux, S., Adriaenssens, E.M., Dutilh, B.E., Koonin, E.V., Kropinski, A.M., Krupovic, M., Kuhn, J.H., Lavigne, R., Brister, J.R., Varsani, A. and Amid, C., 2019. Minimum information about an uncultivated virus genome (MIUViG). Nature biotechnology, 37(1), p.29.).