1) Pore size:**0.45 um**

* Higher concentration of metazoans from samples when compared to 0.22 um which had more bacteria - [*Majaneva et al. 2018*](https://www.nature.com/articles/s41598-018-23052-8)
* Supports consistency across habitats - using a 0.22um filter in temperate and arctic location would be more time consuming and challenging given the greater nutrients in the water

2) Filter type:

* Sterivexes - primary choice due to
* Paper**: cellulose nitrate** - [*Spens et al. 2017*](https://besjournals.onlinelibrary.wiley.com/doi/full/10.1111/2041-210X.12683)*…and others like* [*Liang et al. 2013*](https://pubmed.ncbi.nlm.nih.gov/23869402/)

3) Preservation: **ATL buffer - Qiagen**

* Greater consistency of communities across replicates than samples preserved in EtOH or frozen - [*Majaneva et al. 2018*](https://www.nature.com/articles/s41598-018-23052-8)
* Longevity in terms of maintaining good DNA concentrations - *Smithsonian insight*
* Quantity of ATL buffer > 1.5 ml per sterivex…more lysis buffer the better - [*Wong et al. 2020*](https://www.nature.com/articles/s41598-020-77304-7)
* **Alternative: silica beads then freeze** - [*Allison et al. 2021*](https://bmcresnotes.biomedcentral.com/articles/10.1186/s13104-021-05530-x#:~:text=Silica%20gel%20beads%20are%20an,quality%20prior%20to%20DNA%20isolation.)

4) Replicates: **3-6**

* Minimum of 3 replicates per site and at least 2L BUT more water and/or replicates probably better for tropical locations -  [*Kumar et al. 2021*](https://onlinelibrary.wiley.com/doi/full/10.1002/edn3.235)

5) proK - TBD

6) Taq/Master Mix – TBD

* There are indications of differences between polymerases [*Nichols et al. 2017*](https://pgl.soe.ucsc.edu/nichols18.pdf)
* And personal experience/test indicate some mastermixes are more efficient in PCR amplification.
* VW: I’ve had good results with the Qiagen Multiplex Master Mix for metabarcoding PCR and Kapa HiFi Hot Start Ready mix for indexing PCR.

7) Ligation – 2-step fusion PCR

* Review of Strategies – [*Bohman et al. 2021*](https://onlinelibrary.wiley.com/doi/10.1111/1755-0998.13512)
* Two-Step cost effective with less bias to one-step – [*Zizka et al. 2021*](https://onlinelibrary.wiley.com/doi/10.1111/1755-0998.13018)
* Less bias than indexed samples when comparing a non-fusion 2-step protocol – [*O’Donnell et al. 2016*](https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0148698)
* Less tag jumping than adapter indexed samples
* More manageable workflow
  + all samples have same primers for 1st PCR
  + samples distinguished from i7 and i5 indices from 2nd PCR…no pooling