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Protocol status: In development We are still developing and optimizing this protocol

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© eDNA sample collection method comparison

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ABSTRACT

There is an urgent global need for novel approaches to monitor our freshwater ecosystems. Current biomonitoring tools are time-consuming, costly, and dependent on taxonomic specialists. Therefore, traditional methods are falling short of fulfilling the growing public and policy obligations to monitor these vital, and fragile, ecosystems. Molecular approaches have the potential to revolutionize freshwater biomonitoring but are limited by incomplete reference databases. Furthermore, method development and testing has been lacking for benthic macroinvertebrate and fish environmental DNA (eDNA) surveys. A comparison of eDNA collection and extraction methods is initiated.

PROTOCOL REFERENCES

Elbrecht, V., & Leese, F. (2017). Validation and Development of COI Metabarcoding Primers for Freshwater Macroinvertebrate Bioassessment. *Frontiers in Environmental Science*, *5*(APR), 11. https://doi.org/10.3389/fenvs.2017.00011

Jeunen G-J, Knapp M, Spencer HG, et al. Species-level biodiversity assessment using marine environmental DNA metabarcoding requires protocol optimization and standardization. *Ecol Evol.* 2019; 9: 1323–1335. https://doi.org/10.1002/ece3.4843

Jeunen, G. J., von Ammon, U., Cross, H., Ferreira, S., Lamare, M., Day, R., Treece, J., Pochon, X., Zaiko, A., Gemmell, N. J., & Stanton, J. A. L. (2022). Moving environmental DNA (eDNA) technologies from benchtop to the field using passive sampling and PDQeX extraction. *Environmental DNA, 4*(6), 1420–1433. https://doi.org/10.1002/EDN3.356

Leray, M., Yang, J.Y., Meyer, C.P. *et al.* A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Front Zool* **10**, 34 (2013). https://doi.org/10.1186/1742-9994-10-34

Olmedo-Rojas P, Jeunen G-J, Lamare M, et al. Soil environmental DNA metabarcoding in low-biomass regions requires protocol optimization: a case study in Antarctica. *Antarctic Science*. 2023;35(1):15-30. doi:10.1017/S0954102022000384

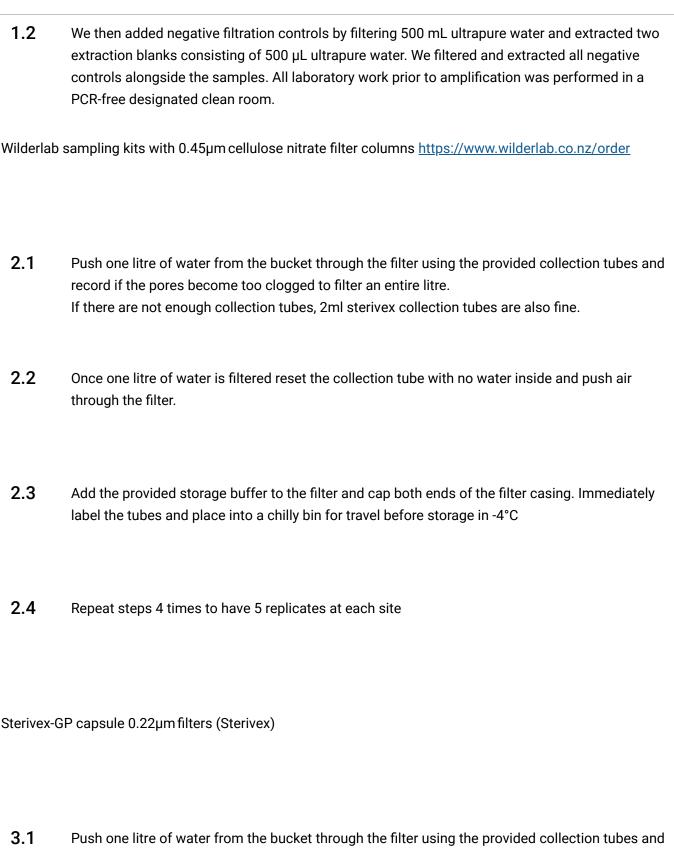
Spens, J., Evans, A.R., Halfmaerten, D., Knudsen, S.W., Sengupta, M.E., Mak, S.S.T., Sigsgaard, E.E. and Hellström, M. (2017), Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods Ecol Evol, 8: 635-645. https://doi.org/10.1111/2041-210X.12683

Sample collection and preservation

- Prior to sample collection, all equipment possible was sterilized using a 10-min exposure to 10% bleach solution and then rinsed with ultrapure water (UltraPureTM DNase/RNase-Free Distilled Water, InvitrogenTM) twice to remove any DNA contamination.
 - Making sure to not handle any equipment involved with sampling without gloves.
 - At each site for each sampling method collect 5 samples at each site. For water it is prefereable that all the water is collected at once in one bucket.
 - 1.1 Water samples must be collected first to minimise sediment in the water column which will clog the filters

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3



record if the pores become too clogged to filter an entire litre.

4

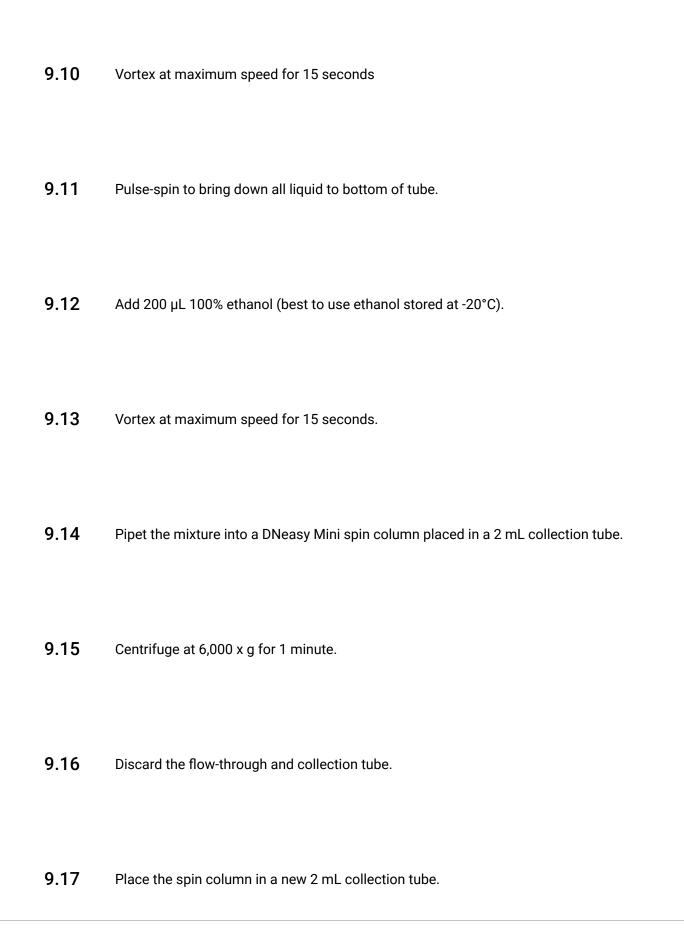
- 3.2 Once one litre of water is filtered reset the collection tube with no water inside and push air through the filter. 3.3 Add Longmire buffer to preserve the DNA in the filter and cap and parafilm the ends of the filter casing. Immediately label the tubes and place into a chilly bin for travel before storage in -4°C 3.4 Repeat steps 4 times to have 5 replicates at each site Vacuum filtration 1.0µm cellulose nitrate filters by a vacuum pump 4.1 Pump one litre of water from the bucket through the filter using an active filtration pump and record if the pores become too clogged to filter an entire litre. 4.2 Once one litre of water is filtered, filters are rolled up using decontaminated tweezers, cut into ~1 mm slices and placed in 2 mL Eppendorf tubes. Immediately label the tubes and place into a chilly bin for travel before storage in -4°C
 - **4.3** Repeat steps 4 times to have 5 replicates at each site
- Only once all water samples have been collected can sediment be then collected to avoid clogging of the filters due to sediment in the water column.
- A
- **6** Sediment samples were collected at the same sites as water samples

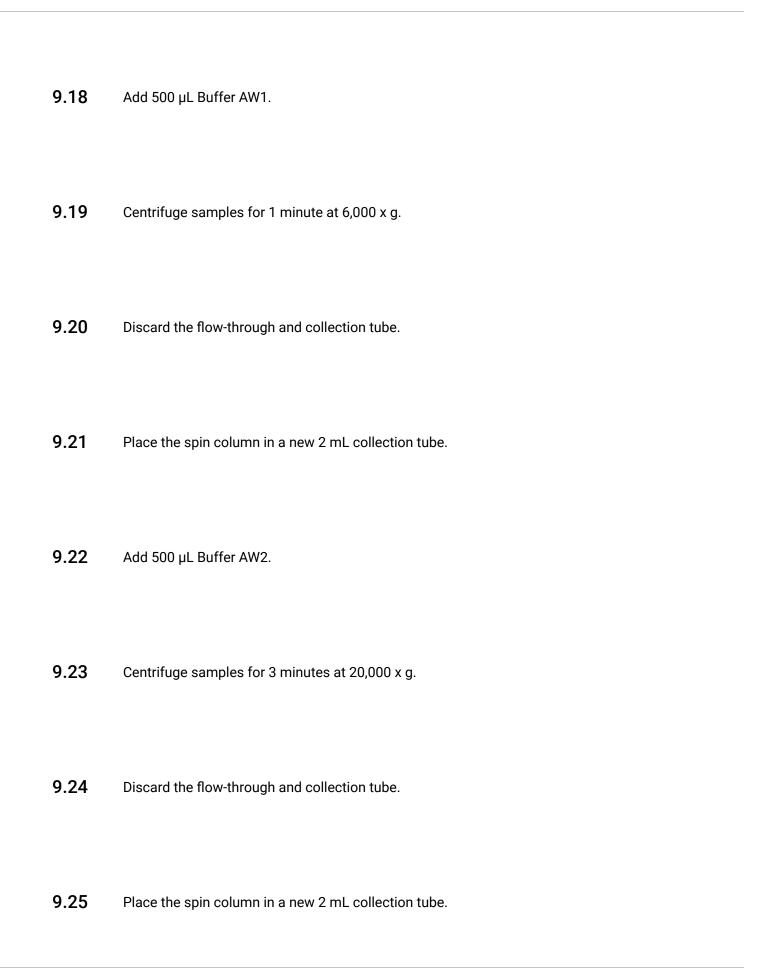
- 6.1 Use 50ml falcon tubes to collect 3 samples of sediment at each site. 6.2 Immediately label the tubes and place into a chilly bin for travel before storage in -4°C. Whirl-Pak® Speci-Sponge® were deployed at the same sampling sites and left overnight 7.1 Using decontaminated chicken wire mesh the sponges were attached using zip ties and deployed at the sites so they were completely submerged and held down with rocks overnight. 7.2 These were then collected, and all samples were kept at -4°C until extraction.
- **8** Physico-chemical water tests were done using YSI Pro2030 Dissolved Oxygen and Conductivity Meter and collecting 50ml falcon tubes for nitrate/nitrite analysis.

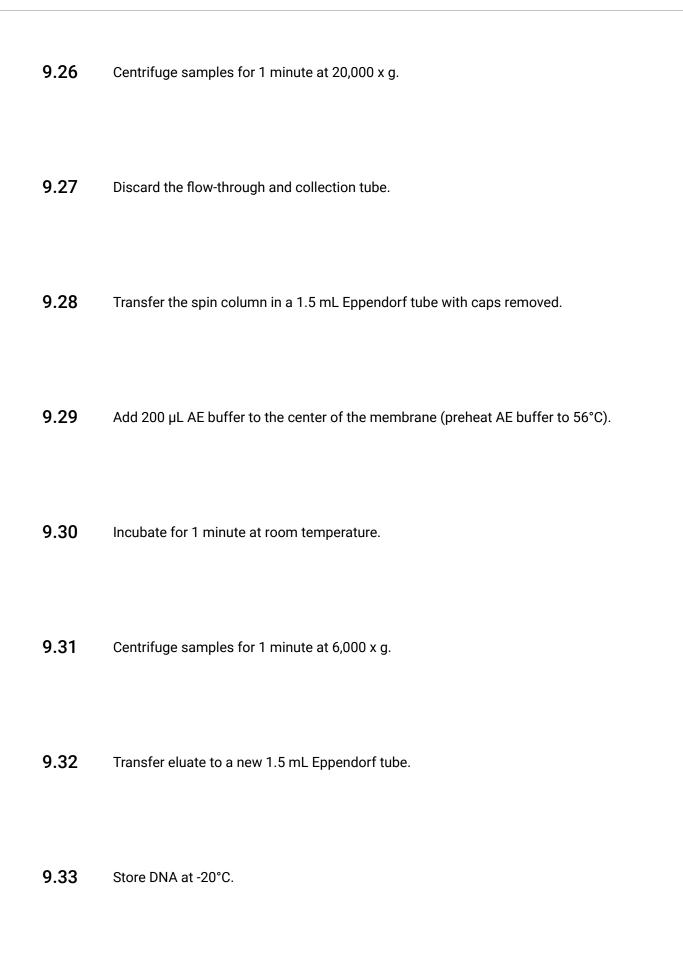
DNA extractions water samples all done using the Qiagen DNeasy Blood &...

9 Wilderlab samples and negative controls extracted as in commercial supplier's instructions

9.1 Use syringe to take out storage buffer from the inlet (don't push the liquid through the outlet!). 9.2 Transfer buffer to a 1.5 mL Eppendorf tube. 9.3 Add 180 μ L ATL buffer and 20 μ L Proteinase K. 9.4 Vortex at maximum speed for 5 seconds. 9.5 Pulse-spin to bring down all liquid to bottom of tube. 9.6 Incubate samples at 56°C overnight. 9.7 Vortex at maximum speed for 5 seconds. 9.8 Pulse-spin to bring down all liquid to bottom of tube. 9.9 Add 200 μL AL buffer.





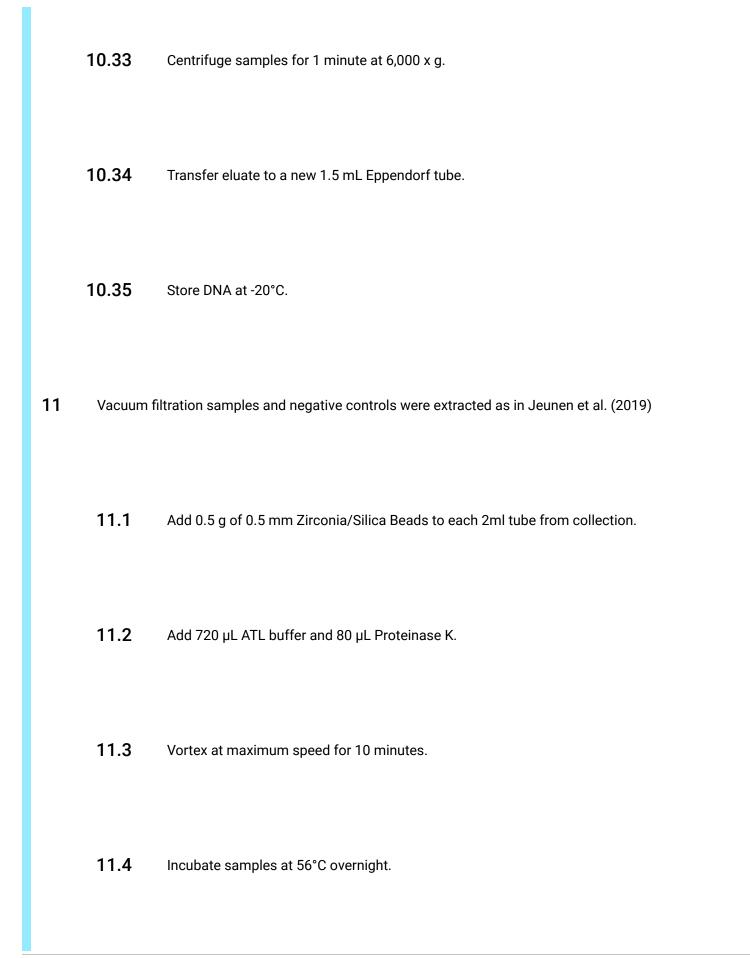


10	Sterivex sa	amples and negative controls extracted using the protocol developed by Spens et al. (2017)
	10.1	Use 5 mL syringe to take out Longmire's buffer from the inlet (don't push the liquid through the outlet!).
	10.2	Transfer buffer to a 1.5 mL or 2 mL Eppendorf tube.
	10.3	Spin buffer at 6,000 x g for 30-45 minutes.
	10.4	Discard supernatant (be careful not to disturb the pellet!)
	10.5	Add 180 μL ATL buffer and 20 μL Proteinase K.
	10.6	Vortex at maximum speed for 5 seconds.
	10.7	Pulse-spin to bring down all liquid to bottom of tube.



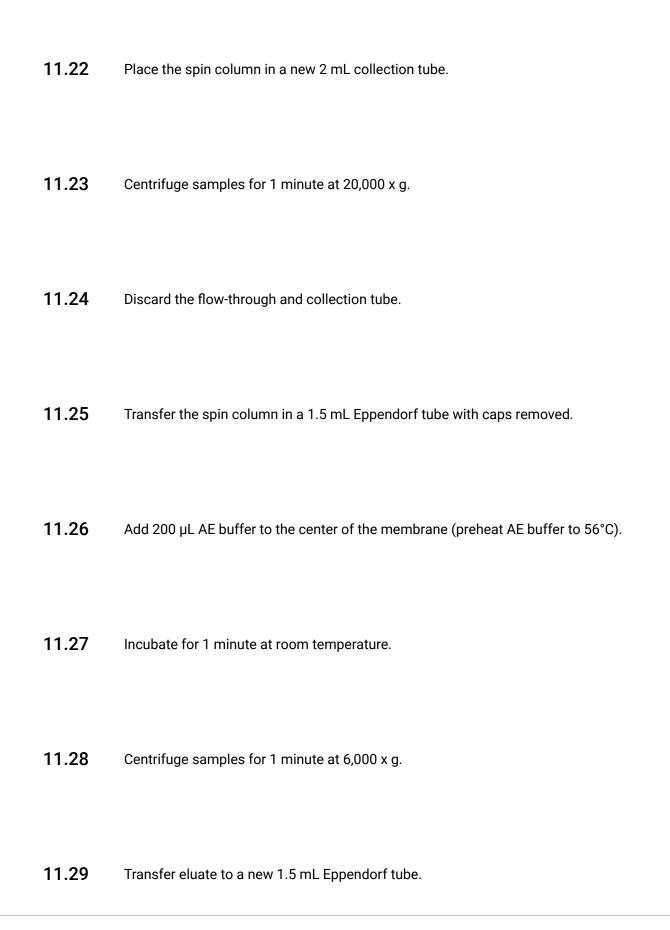
10.17	Centrifuge at 6,000 x g for 1 minute.
10.18	Discard the flow-through and collection tube.
10.19	Place the spin column in a new 2 mL collection tube.
10.20	Add 500 μL Buffer AW1.
10.21	Centrifuge samples for 1 minute at 6,000 x g.
10.22	Discard the flow-through and collection tube.
10.23	Place the spin column in a new 2 mL collection tube.
10.24	Add 500 µL Buffer AW2.

10.25	Centrifuge samples for 3 minutes at 20,000 x g.
10.26	Discard the flow-through and collection tube.
10.27	Place the spin column in a new 2 mL collection tube.
10.28	Centrifuge samples for 1 minute at 20,000 x g.
10.29	Discard the flow-through and collection tube.
10.30	Transfer the spin column in a 1.5 mL Eppendorf tube with caps removed.
10.31	Add 200 µL AE buffer to the center of the membrane (preheat AE buffer to 56°C).
10.32	Incubate for 1 minute at room temperature.



11.5	Vortex at maximum speed for 1 minute.
11.6	Centrifuge samples at 6,000 x g for 1 minute.
11.7	Transfer supernatant (600 μL) into a new 2 mL Eppendorf tube.
11.8	Add 600 μL AL buffer.
11.9	Add 600 μL 100% ethanol.
11.10	Vortex at maximum speed for 1 minute.
11.11	Pipet the mixture (620 μL) into a DNeasy Mini spin column placed in a 2 mL collection tube.
11.12	Centrifuge at 6,000 x g for 1 minute.

11.13	Discard the flow-through and collection tube.
11.14 11.15	Place the spin column in a new 2 mL collection tube. Add 500 µL Buffer AW1.
11.16	Centrifuge samples for 1 minute at 6,000 x g.
11.17 11.18	Discard the flow-through and collection tube. Place the spin column in a new 2 mL collection tube.
11.19	Add 500 μL Buffer AW2.
11.20	Centrifuge samples for 3 minutes at 20,000 x g.
11.21	Discard the flow-through and collection tube.



11.30 Store DNA at -20°C.

DNA extractions sediment samples done using the PowerSoil® DNA Isolat...

- 12 The sediment samples were extracted using a modified extraction protocol without saturated phosphate buffer developed by Olmedo-Rojas et al. (2023)
 - **12.1** Add 0.25 grams of soil sample to a PowerBead Tube
 - 12.2 Vortex for 2 sec
 - 12.3 Add 60 μ L of solution C1 (if solution C1 is precipitated, heat solution to 60°C until dissolved before use)
 - **12.4** Vortex in a horizontal position at maximum speed for 10 mins
 - 12.5 Centrifuge at 10,000 x g for 30 s at ambient temperature
 - **12.6** Transfer the supernatant to a clean 2 ml Collection tube

12.7	Add 250 μL of solution C2 and vortex for 5 s
12.8	Incubate at 4°C for 5 mins
12.9	Centrifuge at room temperature for 1 min at 10,000 x g
12.10	Transfer up to, but no more than, 600 µL of supernatant to a 2 ml Collection tube
12.11	Add 200 μL of Solution C3
12.12	Vortex
12.13	Incubate at 4°C for 5 mins
12.14	Centrifuge at room temperature for 1 minute at 10,000 x g

12.15	Transfer up to, but no more than, 750 µL of supernatant to a 2 ml Collection tube
12.16	Add 1200 μL of Solution C4 to the supernatant (shake Solution C4 before use)
12.17	Vortex for 5 mins
12.18	Load approximately 675 μL onto a Spin Filter
12.19	Centrifuge at 10,000 x g for 1 min at room temperature
12.20	Discard the flow-through
12.21	Add 675 μL of supernatant to the Spin Filter
12.22	Centrifuge at 10,000 x g for 1 min at room temperature

12.23	Load the remaining supernatant onto the Spin Filter
12.24	Centrifuge at 10,000 x g for 1 min at room temperature
12.25	Add 500 μL of Solution C5
12.26	Centrifuge at room temperature for 30 s at 10,000 x g
12.27	Discard the flow-through
12.28	Centrifuge at room temperature for 1 min at 10,000 x g
12.29	Place the Spin Filter in a 2 ml Collection Tube (avoid splashing Solution C5 onto the Spin Filter)
12.30	Add 100 μL of Solution C6 to the centre of the white filter membrane of the Spin Filter tube

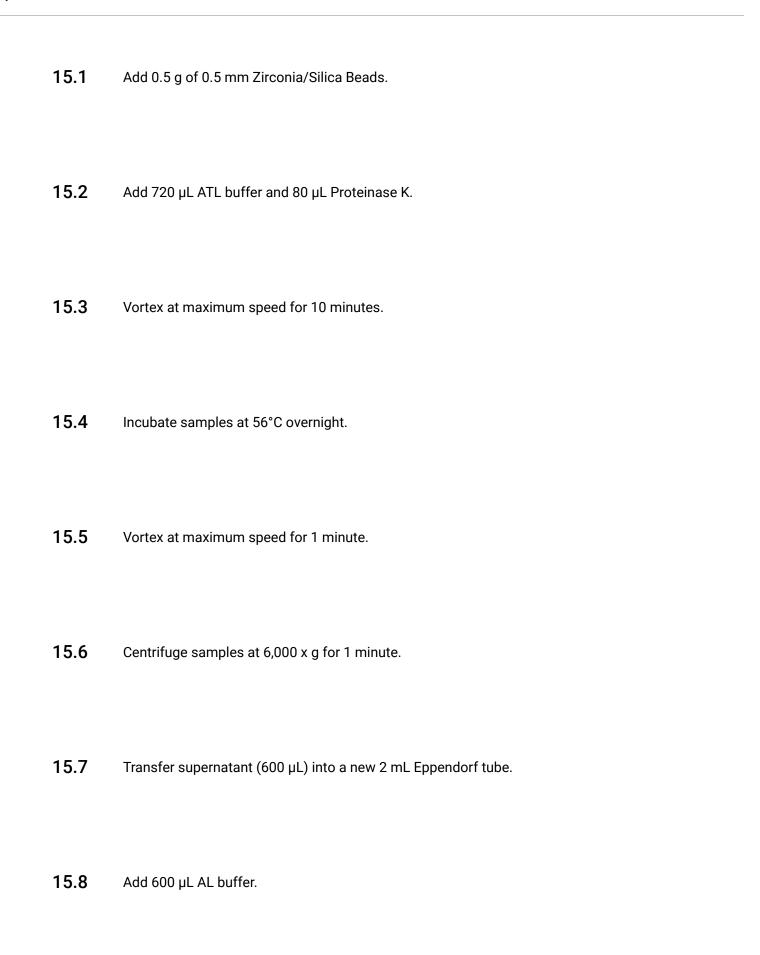
	12.31	Centrifuge at room temperature for 30 s at 10,000 x g
	12.32	Discard the Spin Filter
	12.33	Store DNA at -20° to -80°C
	DV	A section (Court on the court)
	DN	A extractions sponge samples
13	investigat	ction protocol for sponges has not been optimised and so two different extraction techniques were ed. For each of the extractions the sponges were first cut into ten pieces that would each fit into ndorf tubes and then further cut into 3 pieces while in the tube.
14	For the first extraction protocol the tubes were filled with longmire buffer and left to defrost and sit for at least 3 hours.	
	14.1	The longmire buffer was then transferred to a new tube, squeezing out the liquid as much as possible
	14.2	Transfer buffer to a 1.5 mL or 2 mL Eppendorf tube.
	14.3	Spin buffer at 6,000 x g for 30-45 minutes.

14.4	Discard supernatant (be careful not to disturb the pellet!)
14.5	Add 180 μL ATL buffer and 20 μL Proteinase K.
14.6	Vortex at maximum speed for 5 seconds.
14.7	Pulse-spin to bring down all liquid to bottom of tube.
14.8	Incubate samples at 56°C overnight.
14.9	Vortex at maximum speed for 5 seconds.
14.10	Pulse-spin to bring down all liquid to bottom of tube.
14.11	Add 200 μL AL buffer.

14.12	Vortex at maximum speed for 15 seconds
14.13	Pulse-spin to bring down all liquid to bottom of tube.
14.14	Add 200 μL 100% ethanol (best to use ethanol stored at -20°C).
14.15	Vortex at maximum speed for 15 seconds.
14.16	Pipet the mixture into a DNeasy Mini spin column placed in a 2 mL collection tube.
14.17	Centrifuge at 6,000 x g for 1 minute.
14.18	Discard the flow-through and collection tube.
14.19	Place the spin column in a new 2 mL collection tube.
14.20	Add 500 μL Buffer AW1.

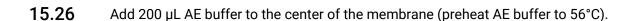
14.21	Centrifuge samples for 1 minute at 6,000 x g.
14.22	Discard the flow-through and collection tube.
14.23	Place the spin column in a new 2 mL collection tube.
14.24	Add 500 μL Buffer AW2.
14.25	Centrifuge samples for 3 minutes at 20,000 x g.
14.26	Discard the flow-through and collection tube.
14.27	Place the spin column in a new 2 mL collection tube.
14.28	Centrifuge samples for 1 minute at 20,000 x g.

14.29	Discard the flow-through and collection tube.
14.30	Transfer the spin column in a 1.5 mL Eppendorf tube with caps removed.
14.31	Add 200 µL AE buffer to the center of the membrane (preheat AE buffer to 56°C).
14.32	Incubate for 1 minute at room temperature.
14.33	Centrifuge samples for 1 minute at 6,000 x g.
14.34	Transfer eluate to a new 1.5 mL Eppendorf tube.
14.35	Store DNA at -20°C.
	second extraction protocol the tubes were left to defrost and the water was removed from the tubes pettes leaving the sponge in the tube before continuing with the extract protocol





15.17	Discard the flow-through and collection tube.
15.18 15.19	Place the spin column in a new 2 mL collection tube. Add 500 μ L Buffer AW2.
15.20	Centrifuge samples for 3 minutes at 20,000 x g.
15.21	Discard the flow-through and collection tube.
15.22	Place the spin column in a new 2 mL collection tube.
15.23	Centrifuge samples for 1 minute at 20,000 x g.
15.24	Discard the flow-through and collection tube.
15.25	Transfer the spin column in a 1.5 mL Eppendorf tube with caps removed.



- 15.27 Incubate for 1 minute at room temperature.
- **15.28** Centrifuge samples for 1 minute at 6,000 x g.
- **15.29** Transfer eluate to a new 1.5 mL Eppendorf tube.
- **15.30** Store DNA at -20°C.

qPCR

Metabarcoding library preparation followed the protocol described in Jeunen et al. (2022). Using universal primers developed by Leray et al. (2013) a qPCR of a dilution series of neat, 10-fold and 100-fold dilution of the input DNA in duplicate must be carried out to ensure adequate amplification and correct for potential PCR inhibitors.

A	В
Component	Vol/15µl reaction
Buffer	12.5 mL mastermix (SensiMix SYBR Lo- ROX Kit – Meridian

Α		В
		Bioscience)
	Forward primer	1 mL
	Reverse primer	1 mL
I	Water	8.5 mL
	DNA	2 mL

A		
PCR programme		
1. 95°C - 10 mins 2. 95°C - 30 secs 3. 50°C - 30 secs50°C - 30 secs 4. 72°C - 150 secs 5. 65°C - 5 mins 45 secs 6.		
6. Back to step 2 x50 cycles in total		

Invertebrate metabarcoding sequencing

Once optimal DNA concentrations are found. Samples can be sequenced using invertebrate specific primers the BF1 and BR2 primers developed by Elbrecht et al. (2017).

Similar to the protocol detailed in Jeunen et al. (2022), a one-step amplification protocol was conducted to generate the amplicons for library preparation using fusion primers containing a modified Illumina sequencing adapter, a barcode tag (6 bp in length), and the template-specific primer.

Barcode sequences were selected with at least three base pair differences to minimize issues with barcode hopping. Each sample was amplified in duplicate and assigned a unique barcode combination to allow for pooling of samples.

17.1 Pool samples into minipools of similar concentrations with the negative controls in a separate minipool.

Measure the DNA concentration of each pool with a high sensitivity qubit assay.

Pool the minipools into one pool at an equimolar concentration. Except the negative controls which just had 1ul added to the final pool.

17.2 Measure the DNA concentration of the final library pool with a high sensitivity qubit assay. Dilute the final library to sequencing specifications.

17.3 Sequence!!!

Fish metabarcoding sequencing

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