FISH DNA extraction:

EcoALpsWater protocol applied for fish DNA extraction from Sterivex cartridge preserved with preservation buffer and using the NucleoSpin® Soil kit (MACHEREY-NAGEL)

Interreg Alpine Space Eco-AlpsWater project – WP1

WPT1 Innovative monitoring approaches (environmental DNA and metabarcoding protocols)
Deliverable D.T1.1.2 Identification and formalization of protocols for eDNA analysis

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I- CONTEXT & GENERAL DESCRIPTION

This protocol is part of the DNA workflow applied in the Eco-AlpsWater Project.

The methodological step described here is the extraction of DNA, this is a critical step for obtaining relevant results since molecular inventories might be influenced by the DNA extraction method used.

The choice of the methodology for fish DNA extraction is based on previous studies and in particular on the work done by (Pont et al. 2018), but adapted to Sterivex cartridges.

For the Eco-AlpsWater project, eDNA fish water sampling are collected with filtration cartridge filled with preservation buffer at RT. The extraction should be done within 1 month after the sampling.

The DNA extraction protocol presented below is based on a protocol adapted from the NucleoSpin® Soil kit (MACHEREY-NAGEL) with specific modifications.

II. DNA EXTRACTION MATERIAL LIST

- Samples (Sterivex cartridge filled with 2mL of preservation buffer)
- NucleoSpin® Soil kit (MACHEREY-NAGEL)
- Sterile 10 ml syringe (2 per sample)
- ATL buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen) (2 x 720 µL per sample)
- Absolute ethanol: 4.4 mL per sample

- Proteinase K: 2 x 20 µL per sample
- Sodium acetate solution (3 M): 200 µL per sample
- Tube shaker
- A centrifuge for 1.5 to 2 mL tubes (relative centrifugal force needed: 11,000 to 15,000 x g)
- A refrigerant centrifuge for 15 mL to 50 mL tubes (relative centrifugal force needed: 15,000 x g and a temperature of 4/6°C)
- Freezer (-20°C)
- Vortex
- Incubator (56°C)
- Gloves
- 1000 µL pipette + tips 1000 µL
- 200 µL pipette + tips 200 µL
- 50 mL sterile tube : 1 per sample
- 15 ml conical tube: 1 per sample
- 2 mL sterile microcentrifuge tubes: 4/5 per sample
- 1.5 or 2 mL sterile microcentrifuge tubes to collect DNA at the end of extraction: 1 per sample
- Parafilm

III. STEPWISE PROTOCOL

III-1 Precautions before sampling

The following precautions must be applied:

- Wear gloves throughout the extraction process.
- Clean the bench with DNA off or 10% commercial bleach before and after manipulation.
- Use tips with filters to avoid contaminations.
- **All steps have to be performed under a specific DNA-work station (sterile area equipped with air filtration and UV systems).**
- **If possible work in a room equipped specifically for rare/degraded DNA** (i.e. room equipped with positive air pressure, UV treatment, frequent air renewal, and accessible only with full protective clothing and mask). In any case the extraction cannot be done in a room were PCR products are amplified.
- ATL buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen) must be heated to 56°C if it precipitates.

III-2 FISH DNA extraction procedure

1. Prepare the sample
    - Agitate the Sterivex containing the preservation buffer for 15 min on a flask shaker at 800 rpm.
2. Lysis Steps

    There are two lysis steps, one from the preservation buffer (PB lysis) and another from the membrane in the empty Sterivex after collection of the preservation buffer (STRX lysis). These two lysis steps are done in parallel and then pooled (Figure 1).

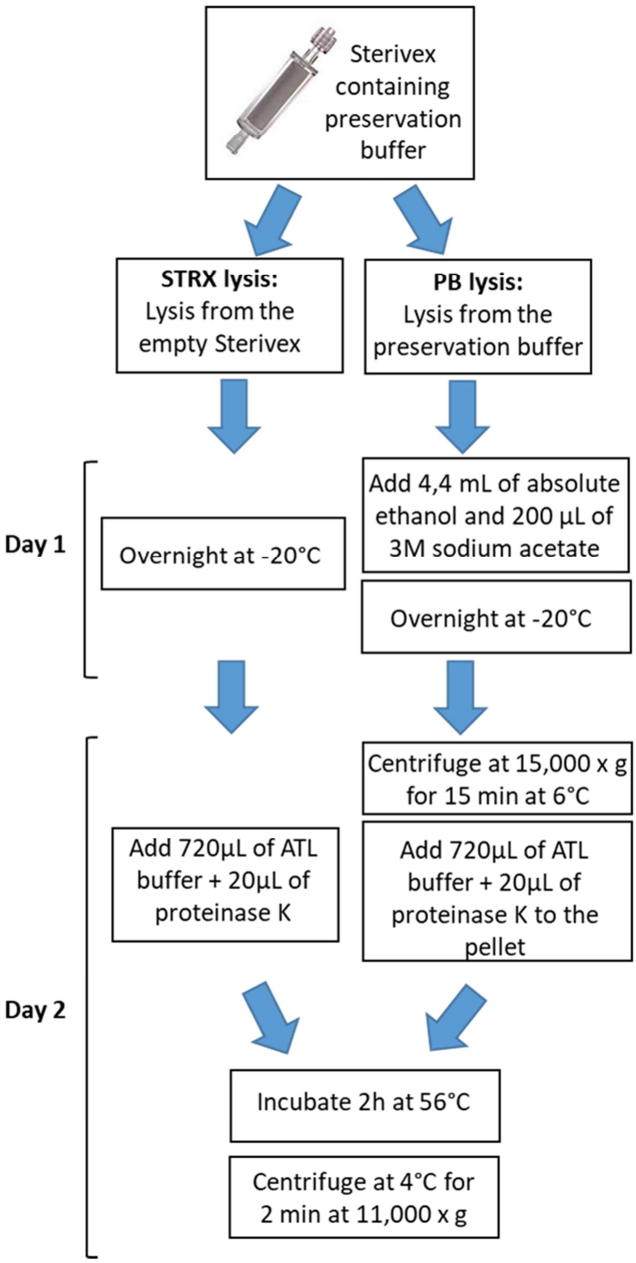

Figure 1: Schematic representations of the two steps of lysis.

- (PB lysis) Remove the Inlet Cap and fix a sterile 10 ml syringe to the Sterivex.
- (PB lysis) Remove the Outlet Cap and aspirate the liquid contained in the Sterivex with the syringe and transfer the lysate from the syringe to a 15 ml conical tube.
- (PB lysis) Add 4.4 mL of absolute ethanol and 200 µL of 3M sodium acetate to the 15mL tube containing the preservation buffer.
- (PB lysis) Store the tube one night at -20°C.
- (STRX lysis) Re-cap the Outlet and the Inlet Cap, and store the empty Sterivex one night at -20°C.

- **The next day**, turn on the incubator at 56°C and the centrifuge at 4°C.
- (PB lysis) take the 15mL tube stored at -20°C, and centrifuge it at 15,000 x g for 15 min at 4°C.
- (PB lysis) Discard the supernatant.
- (PB lysis) Add 720 µL of ATL buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen) to the pellet and vortex.
- (PB lysis) Transfer the mixture to a 2 mL tube containing 20 µL of Proteinase K and mix by pipetting.
- (STRX lysis) Remove the Inlet Cap of the empty Sterivex and add 720 µL of ATL buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen) and 20 µL of Proteinase K. Re-cap the Inlet Cap.
- (PB lysis & STRV lysis) Incubate 2 hours at 56°C with agitation.
- (STRX lysis) Handshake the Sterivex 5 times. Remove the Inlet Cap and fix a sterile 10 ml syringe to the Sterivex. Remove the Outlet Cap and aspirate the liquid contained in the Sterivex with the syringe and transfer the lysate from the syringe to a 2 ml conical tube.
- (PB lysis & STRX lysis) Take the 2mL tubes and centrifuge it for 2 min at 11,000 x g.
- Collect the supernatant contained in each 2mL tubes to a new 2mL tube (2 tubes for each sample, one from STRX lysis and one from PB lysis).

3. Adjust binding conditions
    - Add 250 µL of SB Buffer per 2mL tube from step 2.
    - Close the lid and vortex 5 seconds.

4. Bind DNA

Note: This step can be done with QIAvac 24 Plus manifold to save time.

- Place a NucleoSpin® Soil Column (green ring) in a Collection tube (2 mL).
- Load 550 µL of sample onto the column.
- Centrifuge 1 min at 11,000 x g.
- Discard the flow through and place the column back into the collection tube.
- Load the remaining sample onto the column.
- Centrifuge for 1 min at 11.000 x g.

- Discard the flow through and place the column back into the collection tube. A single column is used to collect DNA from all the 2 mL tubes from one sample.

Note: If a large pellet accumulates in the column and blocks the passage of the sample, resuspend the pellet in SB buffer and recentrifuge the column.

5. Wash and dry silica membrane

Note: the same collection tube is used throughout the entire washing procedure to reduce plastic waste
Note: Heat the SE buffer at 37°C

- 1st wash:
  - Add 500 µL of Buffer SB to the NucleoSpin® Soil Column.
  - Centrifuge for 30 seconds at 11.000 x g.
  - Discard the flow through and place the column back into the collection tube.
- 2nd Wash:
  - Add 550 µL of Buffer SW1 to the NucleoSpin® Soil Column.
  - Centrifuge for 30 seconds at 11.000 x g.
  - Discard the flow through and place the column back into the collection tube.
- 3rd Wash:
  - Add 650 µL of Buffer SW2 to the NucleoSpin® Soil Column.
  - Close the lid and vortex for 2 seconds.
  - Centrifuge for 30 s at 11.000 x g.
  - Discard the flow through and place the column back into the collection tube.
- 4th Wash:
  - Add 650 µL of Buffer SW2 to the NucleoSpin® Soil Column.
  - Close the lid and vortex for 2 seconds.
  - Centrifuge for 30 s at 11.000 x g.
  - Discard the flow through and place the column back into the collection tube.

6. Dry silica membrane
   - Centrifuge for 2 min at 11.000 x g.

   Note: if for any reason, the liquid in the collection tube has touched the NucleoSpin® Soil Column after the drying step, discard flow through and centrifuge again.

7. Elute DNA
   - Place the NucleoSpin® Soil Column into a new microcentrifuge tube (not provided in the kit).
   - Add 30 µL of Buffer SE previously heated to 37°C to the column.
   - Do not close the lid and incubate for 1 min 30 seconds at room temperature (18-25°C).
   - Close the lid and centrifuge for 30 seconds at 11.000 x g.
   - Perform a second elution on the same column: add 30 µL of Buffer SE previously heated to 37°C to the column.

- Do not close the lid and incubate for 1 min 30 seconds at room temperature (18-25°C).
- Close the lid and centrifuge for 30 seconds at 11.000 x g.
- Throw the column and keep the tube containing the DNA.

III-3 Inhibition test and DNA Quantification

After the DNA extraction the samples should be tested for inhibition by qPCR. If the sample is considered inhibited, it should be diluted (5-fold to 10 fold) before the amplification.

Different protocols/equipment can be used to quantify the total amount of DNA extracted:

-Qubit ®Fluorometer system
- Nanodrop
- Spectrofluorimetry

III-4 Storage of DNA working stocks

We recommend storing DNA frozen at -20°C until preparation of DNA library for HTS (or at - 40°C to -80°C for longer storage).

IV References:

NucleoSpin® Soil kit (MACHEREY-NAGEL) - User Manual
https://www.mn-net.com/Portals/8/attachments/Redakteure_Bio/Protocols/Genomic%20DNA/UM_gDNASoil.pdf

Pont, D., M. Rocle, A. Valentini, R. Civade, P. Jean, A. Maire, N. Roset, M. Schabuss, H. Zornig, and T. Dejean. 2018. "Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation." *Scientific Reports* 8. doi: 10.1038/s41598-018-28424-8.