Project BIOLAWEB

Deliverable D4.2

Protocols for molecular tools for macrophytes

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Quality control

Activity	Name	Date
Created	Dr Andreas Ballot, Dr Aleksandra	25/04/2025
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Deliverable summary

Deliverable D 4.2 describes the Protocols for molecular tools for macrophytes for the BIOLAWEB project. It summarizes the steps for sampling, extraction, PRC amplification and sequencing of charophyte/macrophyte eDNA from surface waters. The most important steps and guidelines, as well as links for the official protocols are provided, to ensure consistency, accuracy and reproducibility when sampling eDNA from macrophytes/charophytes for molecular analysis and molecular analysis.

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eDNA sampling – PAF device

Water samples are taken in sterile plastic bottles as 1.5 L integrated water column samples (covering the photic zone), along the transect of the lake, one at each third of the transect. The three samples are later mixed to produce the composite sample to be filtered. The composite water samples are filtered in the field immediately after the sampling, to avoid degradation of DNA, using the Pressure Assisted Filtration system (PAF) *and the eDNA sampling kit, including Sterivex filter* (NIRAS A/S, Allerød, Denmark)*. The protocol is provided by Niras together with the device and includes all the steps for the filtration.*

Description of the steps:

- Mix the 1.5 L water samples in one 5 L container
- Filter the water using the PAF device following the filtration guide, Pressure Assisted Filtration (Kit B) v. 2.9 provided by Niras
- Water is filtered through a 0.45 µm Sterivex filter for max. 30 min or until the filter is clogged
- Preserve the sample by adding 720 µl ATL buffer (Qiagen, Oslo, Norway) in the Sterivex filter and seal it following the protocol in Spence et al. 2017
- Mark the sample and store it for short-term storage at room temperature and for long-term storage at 4°C until further analysis.

eDNA extraction

DNA extraction was conducted according to Spence et al. 2017.

The DNeasy® Blood & Tissue kit (Qiagen, Oslo, Norway) was used as the final step. The Protocol: Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol) was followed, starting from step 4.

The DNeasy® Blood & Tissue kit (Qiagen) protocol is available online at the following address:
https://www.qiagen.com/am/resources/download.aspx?id=68f29296-5a9f-40fa-8b3d-1c148d0b3030&lang=en

It is important to avoid any contamination. All the steps should be done under a captair hood, using gloves, and sterile pipette tips with filters.

The extracted DNA was stored at -20°C until further analysis.

BEFORE STARTING

Material and captair hood preparation

- Clean the station using ethanol or DNA away
- Run UV lights for 15 minutes
- Put the oven at 56°C

PROTOCOL

1. Sample lysis

- Add 80µL of proteinase K (Qiagen) in the Sterivex filter
- Seal the Sterivex with parafilm and set it in a 50 ml Falcon tube
- Incubate at 56°C 24h with low agitation

- After 24h incubation, take the samples out of the oven
- Handshake Falcon tubes five times
- Transfer the entire solution of each Sterivex to 5 mL LoBind tubes using a syringe
- Measure the volume - if it is greater than 1.6 mL, transfer the rest to another LoBind tube
- Add AL buffer + ice-cold EtOH to the sample with the ratio 1:1:1
- Vortex vigorously

In the following steps, the *Qiagen DNAeasy Blood and Tissue kit Protocol: Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol) is used starting at step 4.*

2. Binding step

- Pipette the mixture (650 µL at a time) into a DNeasy Mini Spin column (in a 2 mL collection tube), both provided in the kit
- Spin in micro-centrifuge preferably at 4ºC at 6000 g (8,000 rpm), for 1 min
- Discard the flow-through
- Repeat steps until all sample is filtered through the DNeasy Mini spin column

3. *Washing step*

- Place the DNeasy Mini spin column in a new 2 mL collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard the flow-through and collection tube. *
- Place the DNeasy Mini spin column in a new 2 mL collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard the flow-through and collection tube.

It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.
Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

**All centrifugation steps are carried out at temperature between 20°C and 25°C in a microcentrifuge.*

4. *Elution step*

- Place the DNeasy Mini spin column in a clean 1.5 mL or 2 mL microcentrifuge tube (not provided), and pipette 200 µL Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥ 6000 x g (8000 rpm) to elute.

Elution with 100 µL (instead of 200 µL) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 25 in the online protocol).
Recommended: For maximum DNA yield, repeat elution once.
This step leads to increased overall DNA yield.
A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 7 can be reused for the second elution step.
Note: Do not elute more than 200 µL into a 1.5 mL microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

- Discard the DNeasy spin column
- Store LoBind tube at -20°C or -80°C.

***Reference**:*

Spens, J., Evans, A.R., Halfmaerten, D., Knudsen, S.W., Sengupta, M.E., Mak, S.S., 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 in Ecology and Evolution, 8(5), pp.635-645.
The article is available online at the following address :
https://besjournals.onlinelibrary.wiley.com/doi/pdf/10.1111/2041-210X.12683

PCR - primer pool and dilution

To investigate the presence of Characeae DNA, the primers rbcLanew-F modified after Levin et al. (2003) and rbcLanew-R modified after Kress and Erickson (2007) were selected for rbcl. They cover a sequence length of 553 bp.

These primers are as follows:

Type of primer	Primer Name	Sequence
Forward	rbcLanew-F	*5'-* TGTCACCACARACAGARACTAAARC - *3'*
Reverse	rbcLanew-R	*5'-* GTAAARTCAAGYCCACCRCG - *3'*

Primers are often sold individually at a concentration of 100 µM.
To prepare the primers that are used for the PCR, it is needed to:
Dilute an aliquot of forward and reverse primers at 1/10 to have a concentration of 10 µM required for the PCR.

/!\ *All the following steps need to be done with the maximum precautions to avoid contamination (under a captair hood, with sterile microtubes and molecular grade water)*

***References**:*

Levin, R. A., Wagner, W. L., Hoch, P. C., Nepokroeff, M., Pires, J. C., Zimmer, E. A. & Sytsma, K. J. 2003. Family-level relationships of Onagraceae based on chloroplast rbcL and ndhF data. Am. J. Bot. 90:107–15.
The article is available online at the following address :
https://bsapubs.onlinelibrary.wiley.com/doi/pdfdirect/10.3732/ajb.90.1.107

Kress, W. J. & Erickson, D. L. 2007. A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnH-psbA spacer region. PLoS ONE 2:e508.
The article is available online at the following address :
https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0000508&type=printable

PCR

PCRs can either be conducted as qPCR or normal PCR. In this protocol, qPCRs are used.*

The cycling conditions were as follows:

Phase	Temperature	Time	Cycle
Initialization	95°C	30s	1
Denaturing	95°C	10s	35
Annealing	56°C	20s	
Elongation	72°C	20s	
Melt curve analysis	60-95°C		
End	4°C	∞	

Before PCR, dilute your sample DNA to a maximal concentration of 25 ng/µL.

1. Material and captair hood preparation

- Clean the station using ethanol or DNA away
- Run UV lights for 15 minutes
- Put the reagents at 4°C (water, Ssofast EvagreenTM Supermix, Primer F and Primer R)
- Take the number of PCR strips you will need for the analysis (one well for each sample + one well for a positive control + one well for a negative control). Label them.
- Use 1 microtube of 2 mL for the PCR mix preparation.

2. PCR mix preparation

Considerations before starting: DNA polymerase present in Ssofast EvagreenTM Supermix mix is fragile and all these steps need to be made at low temperature (reagents are kept in the fridge until the last moment, then they are put on ice or in a cold rack under the captair hood)

The table below describes a 25 µL approach.

Reagents	For 1 PCR (25µL)
Water	9 µL
Ssofast EvagreenTM Supermix	12,5 µL
Primer F 10 µM	1.25 µL

Primer R 10 µM	1.25 µL

The used water volume depends on the used volume of DNA (µL).

- Vortex gently all the reagents and do a spin down
- Prepare the mix with the required amount of each reagent according to the table above and vortex at the end
- Put 24 µL of this mix in each PCR strip well (each time, do multiple cycles of aspiration/discharge to be sure the solution is homogeneous)
- Add 1 µL of sample DNA in each well (for the negative control, add nothing)
- Close the lid, agitate gently the well, and do a little spin down.

3. Purification of PCR product

The amplified PCR products were purified using the Monarch® Spin PCR & DNA Cleanup Kit (5 µg) (NEB #T1130) (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol.

Standard Cleanup Protocol using Centrifugation

1. Add 5 volumes (e.g., 250 µL) of Monarch Buffer BZ to 1 volume (e.g., 50 µL) of sample. Mix well by pipetting up and down or flicking the tube. Do not vortex. Using a sample volume of 20-100 µL is recommended. For samples less than 20 µL, adjust the volume with TE or nuclease-free water to 20-100 µL. For diluted samples larger than 800 µL, load 800 µL first, proceed with step 2, and repeat as needed.
2. Insert the Monarch Spin Column S1A into the Monarch Spin Collection Tube and load the sample onto the column.
Spin for 1 minute, then discard the flow-through.
3. Re-insert the column into the collection tube. Wash by adding 200 µL of Monarch Buffer WZ and spin for 1 minute.
Discarding flow-through is optional.
4. Repeat wash (step 3).
5. Transfer the column to a clean 1.5 mL microfuge tube. Use care to ensure that the column tip does not touch the flow-through. If in doubt, re-spin for 1 minute.
6. Add 5-20 µL of Monarch Buffer EY to the centre of the matrix to elute DNA. Wait for 1 minute, and spin for 1 minute.
Nuclease-free water can also be used to elute the DNA. Yield may slightly increase if a larger volume of Monarch Buffer EY is used, but the DNA will be less concentrated. For larger size DNA (≥ 15 kb), incubate the column with elution buffer at room temperature for 5 minutes to maximize the yield. Alternatively, heating the elution buffer to 50°C prior to use can be used.

After purification, DNA concentration was measured using NanoDrop™ 2000 Spectrophotometer (Thermofisher, Waltham, MA USA).
For sequencing with Oxford nanopore kits, the following criteria are needed:
DNA purity (measured using Nanodrop): – OD 260/280 of 1.8 and OD 260/230 of 2.0–2.2
DNA amount: 200 fmol (130 ng for 1 kb amplicons) DNA per sample to be barcoded.

**PCRs for rbcl were performed on a Bio-Rad CFX96 Touch Real-Time PCR Detection System or CFX Opus Real-Time PCR Systems (Bio-Rad Laboratories, Oslo, Norway) using the Ssofast EvagreenTM Supermix (Bio-Rad Laboratories, Oslo, Norway).*

Sequencing

The sequencing is done using the Oxford Nanopore Sequencing Technology.

Barcoding libraries were prepared using the Ligation sequencing amplicons - Native Barcoding Kit 24 V14 (SQK-NBD114.24) according to the protocol of the manufacturer: https://nanoporetech.com/document/ligation-sequencing-gdna-native-barcoding-v14-sqk-nbd114-24
Sequencing was conducted using a Flongle Flow Cell plugged into a MINION device and MinKNOW software (Oxford Nanopore Technologies, Oxford, UK).

Bioinformatic analyses were conducted using the alignment and metagenomics worklow on the EPI2ME platform (Oxford Nanopore Technologies, Oxford UK). Selected Charophyte sequences from BOLD/NCBI databases were used as references.