Project BIOLAWEB

Deliverable D4.1

Protocols for diatoms, phytoplankton

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Reviewed	Dr Frederic Rimet, Dr Miloš Ćirić, Dr Clarisse Lemonnier	06/05/2025
Revised	Dr Danijela Vidaković Dr Miloš Ćirić Kristina Petrović	31/05/2025

Deliverable summary

Deliverable D4.1 describes protocols for diatoms and phytoplankton sampling and metabarcoding analyses performed within the BIOLAWEB project. These protocols provide standardized, step-by-step instructions to ensure consistency, accuracy, and reproducibility of sampling diatoms and phytoplankton organisms for molecular analysis and metabarcoding.

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1. Introduction

Protocols play a crucial role in the field of scientific research, particularly in sample collection and molecular analyses. They provide standardized procedures that ensure the consistency, accuracy, and reliability of results across different laboratories and studies. By outlining specific steps for sample handling, processing, and analysis, protocols help minimize variability and potential errors, enabling researchers to draw valid and reproducible conclusions. In molecular analyses, such protocols are crucial for ensuring the integrity of DNA samples, which are often sensitive to contamination or degradation.

The field protocol and protocol for molecular analysis are adapted from the protocol proposed by Chonova et al. (2021) and Chardon et al. (2020) for the fulfillment of the goals within the BIOLAWEB project.

2. Sampling material list

- Clean nylon brush (e.g., toothbrush), use a different brush for each sampling to avoid contamination;
- Clean plastic spoon or spatula, use a different spoon/spatula for each sampling to avoid contamination;
- A cleaned tray, use a different one for each sample (or clean between each sampling);
- Gloves;
- >90% ethanol (plan 1 L for 10 sapling sites);
- 50 mL plastic, sterile, tube for DNA analysis;
- 50 mL plastic tube for morphological analysis.

3. When and where to sample

3.1 Choice of the sampling season and period

The composition of diatoms and other global phytobenthic communities is changing along with the seasons. In large lakes, the major parameters explaining these temporal changes are nutrients, particularly phosphorus. Oligotrophic communities are observable during summer, characterized by low P concentrations due to microalgae consumption, while mesotrophic communities are present during winter, with higher P concentrations resulting from lake mixing. Moreover, the heterogeneity between the communities present along the shoreline varies from one season to another and is more important in summer (see Lake Geneva in Rimet et al. 2015). However, small or large, but very shallow lakes (e.g., inland saline lakes) are subject to seasonal drying and also to extreme seasonal and daily variations in most environmental parameters (Boros et al. 2013).

For this reason, samplings should be carried out **during summer** in **large lakes**, and **during early spring** in small or large, but very **shallow lakes**.

NOTE: If wind-induced strong waves occur during several days and scoured the biofilms, then you have to wait for 2 weeks before collecting the samples to allow biofilms to restructure.

3.2 Choice of the sampling locations

The number of samples per lake will depend on lake size. For large lakes (e.g., Ohrid Lake, Prespa Lake, Geneva Lake), it is recommended to have at least 10 sites, with samples typically taken every 0.5 to 1.5 km along the shoreline. Since 10 samples are a relatively small number to cover the entire perimeter of a lake, we propose choosing a small stretch of the lake's shoreline. This stretch should be selected by expert judgment and should exhibit known heterogeneity (Figure 1A). For medium-sized lakes (e.g., Sava Lake, Pečena Slatina) (Figure 1B), it is recommended to have at least three sites, and for small and shallow lakes, one site (e.g., Velika Rusanda, Slano Kopovo, Okanj bara) (Figure 1C).

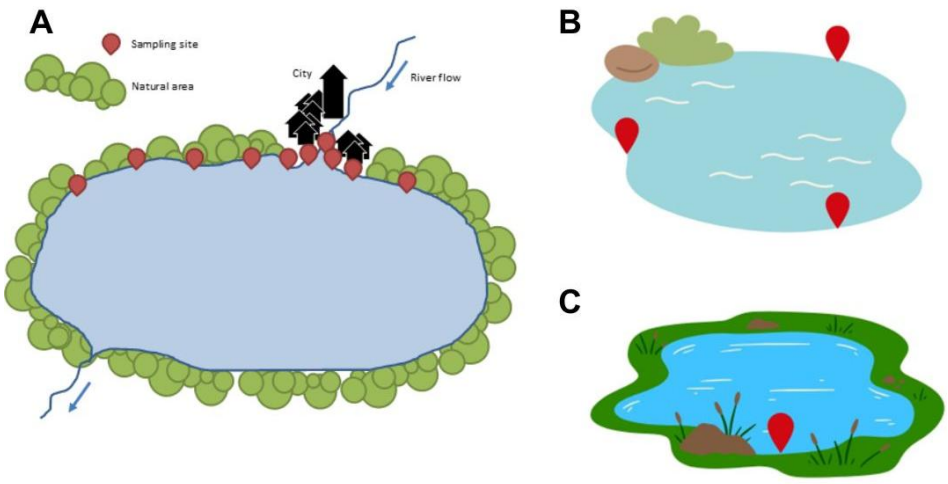

Figure 1. A. Example of the positioning of the 10 sampling sites along the lake's shoreline (figure from Chonova et al. 2021); B. Example of the positioning of the three sampling sites along the lake's shoreline; C Example of the positioning of the one sampling.

4. Biofilms sampling procedure and preservation

Standards such as European Standards EN 13946 and EN 15708 advocate the use of a range of natural and artificial substrates, including rock, mud, sand, plants, glass slides, brick, and tiles, for sampling diatoms (Richards et al. 2020). Nevertheless, the choice of substrate will depend on the nature of the lake bottom being sampled. Protocols for diatom sampling from the most common substrates are explained below.

4.1 Stone

- Take five stones (or more), at 20-40 cm depth in an area of 100 m²;
- Let the stones drain for a few minutes;
- Fill the bottom of the tray with distilled water;
- Brush the stones in the tray;
- Take the biofilm from the tray and fill the tube up to 10 ml;
- Complete the tube with 90% ethanol (or > 90%) up to 45 ml (leave 5 ml of empty space from the top); (Figure 2)
- Shake to homogenize, label;

- The rest of the sample is transferred from the tray to the new plastic tube for morphological analyses.

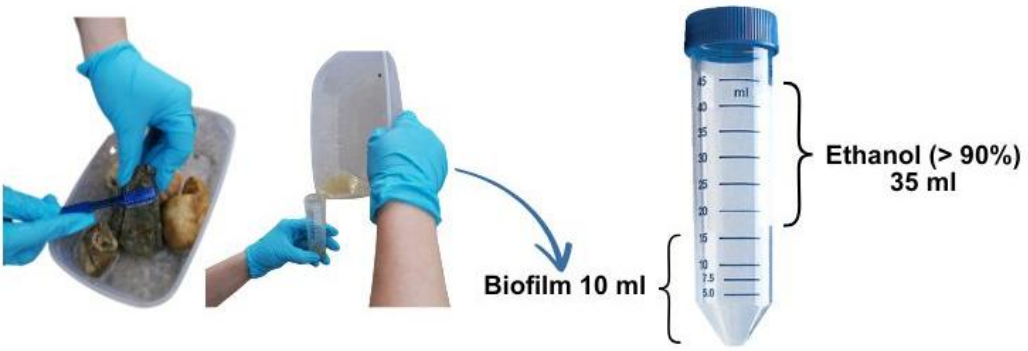

Figure 2. Schematic sampling procedure in the field when stones are available.

4.2 Mud

- Take the surface layer of mud from at least three spots in an area of 100 m²; at 20-40 cm depth;
- Put the mud in the tray, and stir with the plastic spoon or spatula (new for each sample) to homogenize;
- Take the mud from the tray and fill the tube up to 10 ml;
- Complete the tube with 90% ethanol (or> 90%) up to 45 ml (leave 5 ml of empty space from the top); (Figure 3)
- Shake to homogenize, label;
- The rest of the sample is transferred from the tray to the new plastic tube for morphological analyses.

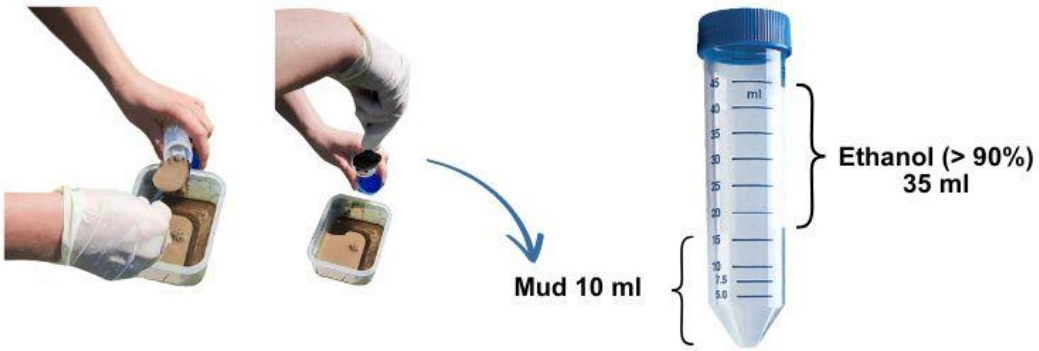

Figure 3. Schematic sampling procedure in the field when mud is available.

4.3 Macrophytes

- Take 5 submerged stems, at 20-40 cm depth;
- Brush the stems in the tray, adding distilled water;
- Take the biofilm from the tray and fill the tube up to 10 ml;
- Complete the tube with 90% ethanol (or> 90%) up to 45 ml (leave 5 ml of empty space from the top) (Figure 4);

- Shake to homogenize, label;
- The rest of the sample is transferred from the tray to the new plastic tube for morphological analyses.

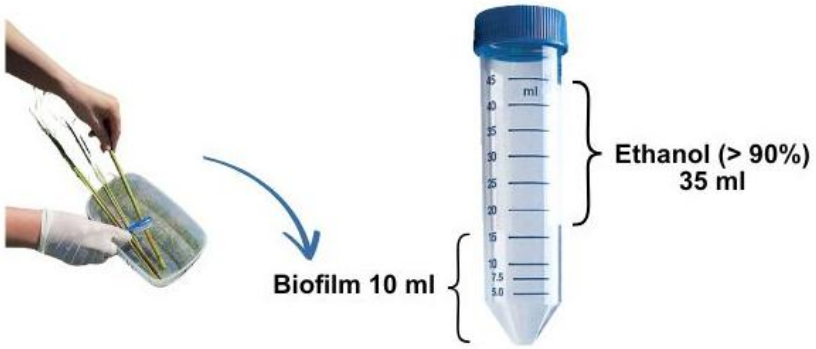

Figure 4. Schematic sampling procedure in the field when macrophytes (etc., Typha) are available.

NOTE: Biofilm for DNA analysis can be frozen immediately after collection, in which case ethanol is not added.

5. DNA extraction

This protocol is based on the Nucleospin Soil kit, adapted for the DNA extraction of diatom biofilms conserved in ethanol. Starting from this point, it is important to avoid any contamination. All the protocol must be done under a captair hood, using gloves, and sterile pipette tips with filters.

There is a version available *online* here: https://www.protocols.io/view/dna-extraction-from-environmental-biofilm-using-th-e6nvw9odzgmk/v1

5.1 Preparation for DNA extraction

Material and captair hood preparation:

- Clean the station using ethanol or DNA away.
- Run UV lights for 15 minutes.
- Put the centrifuge at a temperature of 4°C.

Solution preparation:

- The first solution, SL1, contains SDS that can precipitate at room temperature. Before starting the DNA extraction, look carefully if there is such a precipitate. If so, put the SL1 solution at 30-40°C for 10 minutes, and agitate regularly.
- For the first use of the kit, you need to prepare the SW2 buffer. For this, you must add the required volume of ethanol (96-100%) to the SW2 buffer and note on the bottle that it now contains ethanol. Starting from this point, the SW2 solution is stable at room temperature for at least one year.
- Incubate solution SE at 50°C

5.2 DNA extraction – Protocol

1. Sample preparation:

- Homogenize the sample by shaking the falcon tube vigorously.
- Visually check the density of the biofilm. If there are very dense biofilms, you can cut some tips before (using scissors washed with DNA-away and rinsed with ethanol), in order to be able to aspirate the biofilm.
- Take 2 mL by pipetting 1 mL twice and put it in a 2 mL eppendorf tube.
  Note: between each pipetting, aspirate/discharge to resuspend the biofilm particles that may have sedimented at the bottom of the tube.
- Centrifuge at 18000 g for 30 minutes.
- Remove the supernatant using a pipette.
- This step is ideally done before the DNA extraction. The pellet can be stored at -20°C for weeks.

2. Sample lysis (Figure 5):

- Add 700 µL of SL1 (lysis buffer) to the pellet. Perform multiple cycles of aspiration and discharge to ensure that all the pellet is completely dissolved.
- Put the solution (SL1+biofilm) in a Nucleospin Bead tube A.
- Add 150 µL of solution SX (enhancer solution for cell lysis).
- Vortex for 5 min horizontally at maximum speed.

At this step, cells are lysed using chemicals present in the SL1 solution, such as SDS, and with mechanical lysis.

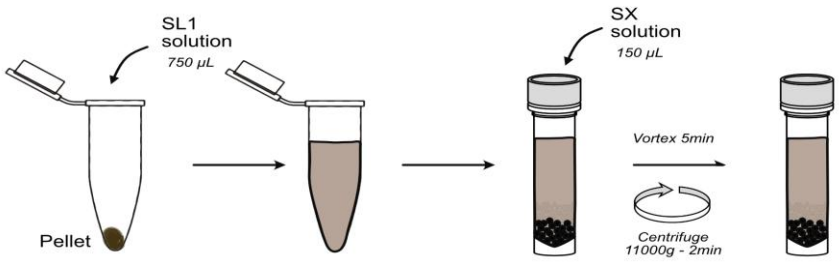

Figure 5. Sample lysis.

3. Precipitate contaminants (Figure 6):

- Centrifuge the Nucleospin Bead tube A at 11 000 g for 2 minutes.
- Add 150 µL of SL3 solution (that reacts with the contaminants). Vortex quickly for 5 seconds.

- Incubate at 4°C (in a fridge) for 5 minutes.
- Centrifuge at 11 000 g for 1 min.

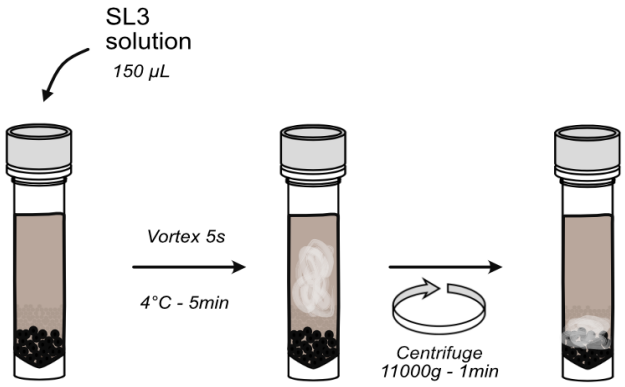

Figure 6. Precipitate contaminants.

4. Remove contaminants (Figure 7):

This step is performed using a column that adsorbs the different contaminants (the column with a red ring from the Nucleospin Soil kit). BE CAREFUL with this step, the DNA will not be retained by the column.

- Place the column in a 2 mL eppendorf tube (not provided by the kit).
- Add 650 µL of clear supernatant (obtained after centrifugation in the previous step).
- Centrifuge at 11,000 g for 1 min.
- Aspirate the solution that has passed through the column (containing the DNA) and transfer it to a new 2 mL tube.
- Re-do the column load (with 650 µL of supernatant) as many times as there is supernatant left to filtrate (theoretically 2 times).
- Discard the column at the end.

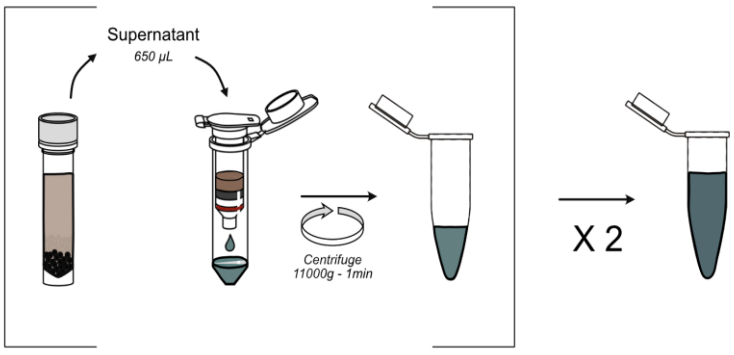

Figure 7. Removing contaminants.

5. Adjust binding conditions (Figure 8):

- Add 250 µL of buffer SB to the tube containing the sample.
- Vortex for 5 seconds and do a brief centrifugation.

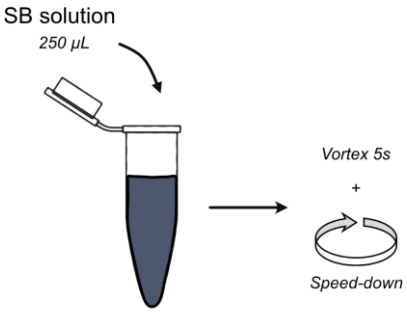

Figure 8. Adjust binding conditions.

6. Fix DNA to the silica membrane (Figure 9):

- Put a Nucleospin Soil column into a 2 mL eppendorf tube (given by the kit).
- Load 650 µL of sample in the column.
- Centrifuge at 11,000 g for 1 min.
- Discard the solution that passed through the column.
- Load the remaining volume of sample in the column.
- Centrifuge at 11,000 g for 1 min.
- Discard the solution that passed through the column.

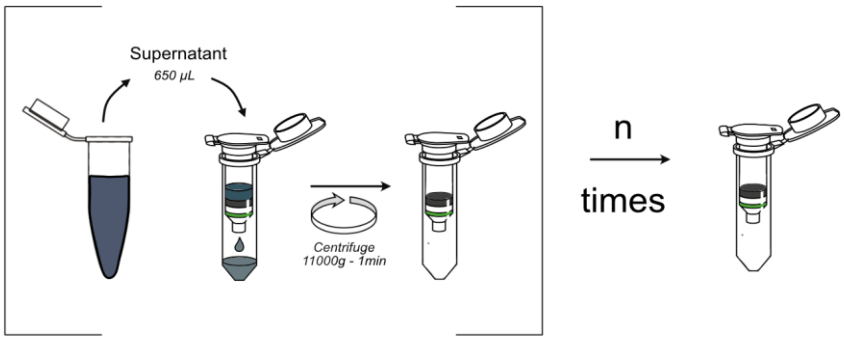

Figure 9. Fix DNA to the silica membrane.

7. Washing of DNA (Figure 10):

1st washing

- Add 500 µL of solution SB.
- Centrifuge at 11,000 g for 30s.
- Discard the flow-through and return the column to the tube.

2nd washing

- Add 550 µL of solution SW1.
- Centrifuge at 11,000 g for 30s.
- Discard the flow-through and place the column back in the tube.

3rd washing

- Add 650 µL of solution SW2.
- Centrifuge at 11,000 g for 30s.
- Discard the flow-through and place the column back in the tube.

3rd washing - bis

- Add 650 µL of solution SW2,
- Centrifuge at 11,000 g for 30s,
- Discard the flow-through and place the column back in the tube.

8. Dry silica membrane (Figure 9):

- Centrifuge at 11,000 g for 2 min.

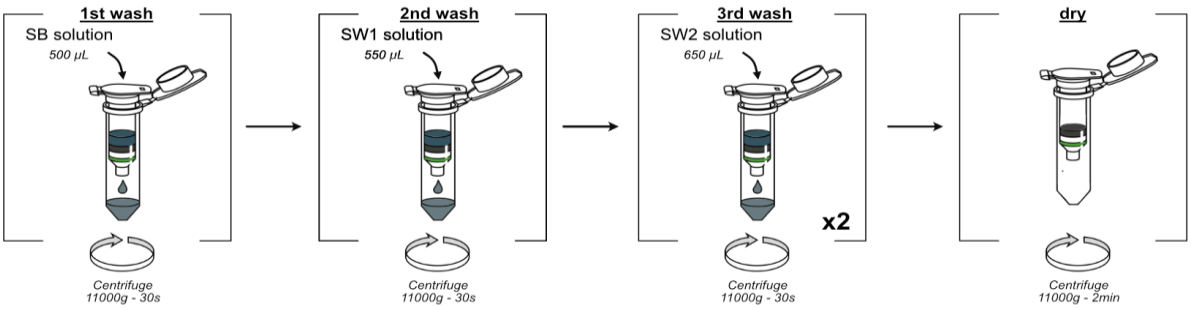

Figure 10. Washing of DNA and Dry silica membrane.

9. DNA elution (Figure 11):

- Place the Nucleospin Soil column in a 1.5 mL eppendorf tube.
- Add 30 µL of SE solution.
- Wait for 1 minute and 30 seconds for the solution to impregnate the column.
- Close the tube and centrifuge at 11,000 g for 30s.
- Discard the Nucleospin Soil column.
- Keep the tube containing DNA at -20°C for downstream analysis.

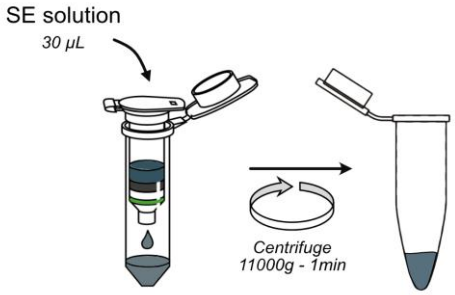

Figure 11. DNA elution.

6. Diatom PCR - primer pool and dilution

This protocol can be found online: https://www.protocols.io/view/diatom-dna-library-preparation-for-illumina-miseq-kqdg3573zv25/v1

Primers for amplifying rbcL in this protocol are Diat_rbcl_708F and R3, which amplify a 312-bp fragment. To maximize the amplification of diatom diversity, we employ a combination of three different forward and two different reverse primer sequences (Vasselon et al. 2017).

These sequences are as follows:

Type of primer	Primer Name	Sequence
Forward	Diat_rbcl_708F_1	*5'*- AGGTGAAGTAAAAGGTTCWTACTTAAA - *3'*
Forward	Diat_rbcl_708F_2	*5'*- AGGTGAAGTTAAAGGTTCWTAYTTAAA - *3'*
Forward	Diat_rbcl_708F_3	*5'*- AGGTGAAACTAAAGGTTCWTACTTAAA - *3'*
Reverse	R3_1	*5'*- CCTTCTAATTTACCWACWACTG - *3'*
Reverse	R3_2	*5'*- CCTTCTAATTTACCWACAACAG - *3'*

Different ways of preparing Illumina libraries for metabarcoding exist. In this protocol, we use a 2-step PCR. The first step will specifically amplify the rbcL fragment from the sample DNA, and the sequencing platform will perform the second step to add the index and other required elements for Illumina sequencing.

To do so, our primers contain Illumina adapters P5 and P7 at the 5' end of the forward and reverse primers, respectively. These adapters will serve as primers for fixation in the second PCR.

Type of primer	Primer Name	Sequence
Forward	Diat_rbcl_708F_1	*5'***CTTTCCCTACACGACGCTCTTCCGATCT**AGGTGAAGTAAAAG GTTCWTACTTAAA - *3'*
Forward	Diat_rbcl_708F_2	*5'***CTTTCCCTACACGACGCTCTTCCGATCT**AGGTGAAGTTAAAG GTTCWTAYTTAAA - *3'*
Forward	Diat_rbcl_708F_3	*5'***CTTTCCCTACACGACGCTCTTCCGATCT**AGGTGAAACTAAAG GTTCWTACTTAAA - *3'*
Reverse	R3_1	*5'***GGAGTTCAGACGTGTGCTCTTCCGATCT**CCTTCTAATTTACC WACWACTG - *3'*
Reverse	R3_2	*5'***GGAGTTCAGACGTGTGCTCTTCCGATCT**CCTTCTAATTTACC WACAACAG - *3'*

Primers are often sold individually at a concentration of 100 µM. So, to prepare the primers that we will use for the PCR, we need to:

- 1/ Pool in an equimolar mix the 3 forward primers
- 2/ Pool in an equimolar mix the 2 reverse primers
- 3/ Dilute this pool at 1/10 to have a concentration of 10 µM required for the PCR

These steps need to be adapted to the number of samples.

Example for ~ 40 samples of diatoms:

/!\ 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).

Preparation of the Forward primer:

- Mix 8 µL of each forward primer in a microtube
- Add 20 µL of this solution to 180 µL of water

Preparation of the Reverse primer:

- Mix 12 µL of each reverse primer in a microtube
- Add 20 µL of this solution to 180 µL of water

7. Diatom PCR

This protocol can be found online here: https://www.protocols.io/view/diatom-dna-library-preparation-for-illumina-miseq-kqdg3573zv25/v1

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

1. Material and captair hood preparation:

- Clean the station using ethanol or a DNA-removing solution.
- Run UV lights for 15 minutes.
- Put the mix reagent at 4°C (water, Buffer, dNTP, BSA, Primer F, and Primer R).
- Determine the number of PCR strips required for the analysis (one well for each sample, plus one for a positive control and one for a negative control). Label them.
- Use a 2 mL microtube for PCR mix preparation.

2. PCR mix preparation:

Considerations before starting: DNA polymerase present in the KAPA HiFi mix is fragile, and all these steps must be performed at low temperatures. Reagents are kept in the fridge until the last moment, then they are placed on ice or in a cold rack under the centrifuge hood.

Reagent	For 1 PCR
Water	15.6 µL (13.1 µL if using the TAQ 125U)
Buffer 10X	2.5 µL
dNTP 2.5 mM	2 µL
BSA 10 mg/mL	1.25 µL
Mg2+ (FOR TAQ 125U ONLY)	2.5 µL
Primer F 10 µM	1.25 µL
Primer R 10 µM	1.25 µL
TaKaRa LA Taq	0.15 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, respecting the order: start with water, then add buffer…, and finally add Taq.
- Add 24 µL of this mix to each PCR strip well (perform multiple cycles of aspiration and discharge to ensure the solution is homogeneous each time).
- Add 1 µL of sample DNA to each well (for the negative control, add no DNA).
- Close the lid, gently agitate the well, and perform a brief spin-down.

3. PCR thermal cycler:

rbcL	Temp in °C	Cycle	Time
Denaturation	95		6 min
Aannealing	54	33	1 min
Elongation	72		6 min

4. PCR revelation

Gel preparation

Prepare the gel according to the table below:

	Agarose (high purity)	TAE Buffer (1%)	Microwave	HD Green Plus DNA Stain*
Small gel	0.60 g	~ 50 mL	1.30 min	3 µL
Big gel	1.80 g	~ 150 mL	2 min	9 µL

*Stored at +4°C

Gel loading and migration

- Place the gel into a gel electrophoresis tank (be sure it is completely immersed; otherwise, add more buffer).
- Load 3 µL of PCR amplicon into the gel using loading buffer.
- Load 3 µL of DNA ladder at the beginning.
- Launch migration at 70V, 90 amperes for 40 min (small chamber) or 165V, 200 amperes for 45 min (big chamber).

Gel revelation

- Take the gel from the electrophoresis tank.
- Put it in the gel reader.
- With « preview » mode, verify if it is placed under the camera.
- Launch UV, stop when the image intensity is correct.
- Register the picture.
- Put the gel in the specific container at the end.

8. Introduction to phytoplankton analysis

The procedure for phytoplankton sampling for metabarcoding analysis was developed within WP4 – Research and partnership of the BIOLAWEB project. The DNA extraction procedure is similar to the one for diatoms, with a slight modification to the lysis step. The recommended marker gene used for metabarcoding of phytoplankton is the 23S ribosomal RNA gene present in chloroplasts and cyanobacteria. The primers, as well as the complete protocol for PCR, were developed by Alexis Canino within the OFB and INRAE project PhytoDOM (Canino et al. 2023).

9. Phytoplankton sampling

9.1 Sampling design (temporal and spatial scale)

Depending on the size of the water body, several samples should be taken along the longitudinal profile of the lake. Within the BIOLAWEB project, phytoplankton samples for eDNA analysis were collected at three sites near the shore (littoral zone) and one central point of each reservoir/pond during the spring, summer, and autumn seasons.

9.2 List of sampling equipment and consumables

- Water sampler (clean plastic hose)
- Plastic bottles (clean, volume 2 L)
- Cool bag with clean plastic containers
- Sterile luer lock syringes large (50 mL)
- Sterile luer lock syringes small (5 mL)
- Sterivex filters
- Sterile luer lock stoppers
- Lysis buffer solution
- Labeled zip-lock plastic bags
- Gloves

9.3 How to collect samples for eDNA analyses?

- Take the water samples for phytoplankton metabarcoding analysis using a plastic hose along the vertical profile covering the photic zone (e.g., two times the Secchi depth)
- Pour the water from the hose into clean sampling bottles and transport them to the shore
- Filter 250 mL of water using Sterivex filter attached to the syringe (50 mL)*
- Remove the water trapped in the Sterivex filter by pushing the air with the empty syringe
- Close one end of the Sterivex filter with the sterile luer lock stopper
- Add 2 mL of lysis buffer to the Sterivex filter using the syringe (5 mL)
- Close the other end of the Sterivex filter with the sterile luer lock stopper
- Transfer the Sterivex filter into the labeled zip-lock bag and place the material into a cool bag

The detailed demonstration of sampling steps created within the INRAE's PhytoDOM project is available at the following link: https://www.youtube.com/watch?v=x_t0-063Qno

*In eutrophic lakes, it is usually challenging to filter 250 mL of water, so it is recommended to filter less until the filter is clogged. Note the filtered water volume.

How to prepare the preservation buffer?

The protocol for preparing phytoplankton preservation buffer solution can be found in the «before starting» section of the following online protocol: https://www.protocols.io/view/fish-edna-water-sampling-and-filtration-through-st-ewov142xyvr2/v1

Table 1. Concentration of preservation buffer reagents and example of volumes to prepare 10 mL and 50 mL of the buffer

Reagent	Final concentration	For 10 mL	For 50 mL
EDTA	40 mM	800 µL of 0.5 M EDTA	4000 µL of 0.5 M EDTA
Tris-HCl (pH 8)	50 mM	500 µL of 1 M Tris-HCl	2500 µL of 1 M Tris-HCl
Sucrose	0.75 M	2.55 g	12.75 g

Lysis buffer preparation steps:*

- Weigh sucrose in a beaker;
- Add EDTA;
- Add Tris-HCl;
- Add ultrapure water (half of the final volume) and dissolve sucrose using a magnetic stirrer;
- Transfer the solution in a volumetric flask, complete with ultrapure water, to the final volume and shake;
- Filter-sterilize solution using a 0.2 µm unit filter;
- Split into 2100 µL aliquots (e.g., sterile Eppendorf tubes) and store in the fridge at 4°C.

*It is important to work in a sterile environment.

10. DNA extraction - Phytoplankton

This protocol is similar to the one for diatoms, except that the lysis step is done directly in the Sterivex filter. It can be found online in the following link: https://www.protocols.io/view/fish-edna- dna extraction-from-water-samples-filter-bp2l6npqdgqe/v1

10.1 Phytoplankton DNA extraction – Protocol

The process of DNA extraction can be divided into several steps:

Material and captair hood preparation

- Clean the station using ethanol or DNA away
- Run UV lights for 15 minutes
- Put one oven at 37°C and one oven at 55°C

Sample preparation

- Let the Sterivexes (if they are frozen) at 4°C for 30 min
- Vortex them horizontally for 5 min at the maximum intensity

Sample lysis

- Add 40 µL of lysozyme to each Sterivex
- Incubate at 37°C for 45 min with agitation (in a rotor)
- Add 50 µL of proteinase K and 100 µL of SDS 20% in each Sterivex
- Incubate at 55°C for 2 h with agitation (in a rotor)

Precipitate contaminants

- Aspirate the entire solution of each Sterivex using a 5 mL syringe and transfer the lysate into two 2 mL tubes (about 1.1 mL per tube)
- Add 200 µL of SL3 solution. Vortex quickly for 5 s
- Incubate at 4°C (in a fridge) for 5 minutes
- Centrifuge at 11,000 g for 1 min

At this step, put the solution SE at 55°C to anticipate the elution step

Remove contaminants

- Put the column (red ring) in a 2 mL Eppendorf tube (this tube is not provided in the kit)
- Add 650 µL of clear supernatant (obtained after centrifugation in the previous step)
- Centrifuge at 11,000 g for 1 min
- Put the column in a new 2 mL tube and close the previous one.
- Repeat these steps as often as there is still some supernatant from step 3 to be filtered. For each centrifugation, collect the supernatant in a clean tube (about 4 tubes are required, but only 1 column for the filtration of all lysate from step 3)
- Discard the column at the end

Adjust binding conditions

- Add 250 µL of buffer SB in each of the 4 tubes containing the sample
- Vortex for 5 seconds and do a brief centrifugation

Fix DNA to the silica membrane

- Place a Nucleospin Soil column (green ring) into a 2 mL Eppendorf tube (given by the kit)
- Load 650 µL of sample in the column
- Centrifuge at 11 000 g for 1 min
- Discard the flow-through and place the column back into the collection tube
- Repeat the three previous steps with all the tubes, for each sample use the same column.

Washing of DNA

1st washing

- Add 500 µL of solution SB
- Centrifuge at 11,000 g for 30 s
- Discard the flow through and place the column back in the tube

2nd washing

- Add 550 µL of solution SW1
- Centrifuge at 11,000 g for 30 s
- Discard the flow through and place the column back in the tube

3rd washing

- Add 650 µL of solution SW2
- Centrifuge at 11,000 g for 30 s
- Discard the flow through and place the column back in the tube

3rd washing - bis

- Add 650 µL of solution SW2
- Centrifuge at 11,000 g for 30 s
- Discard the flow through and place the column back in the tube

Dry silica membrane

- Centrifuge at 11,000 g for 2 minutes

DNA elution

- Place the Nucleospin Soil column in a 1.5 mL Eppendorf tube
- Add 30 µL of SE solution
- Wait for 1 min 30 s for the solution to impregnate the column (do not close the lid)
- Close the tube and centrifuge at 11,000 g for 30 s
- Discard the Nucleospin Soil column
- Keep the tube containing DNA at -20°C for downstream analysis

11. Phytoplankton PCR

An effective identification of phytoplankton, which is usually composed of different eukaryotic and prokaryotic organisms, is based on a common gene marker present in both groups.

11.1 Phytoplankton PCR primers

To investigate phytoplankton diversity, the 23S ribosomal RNA gene present in chloroplasts and cyanobacteria is targeted. Primers were developed by A. Canino under the PhytoDOM project and have been published recently (Canino et al. 2023).

Table 2. Characteristics of phytoplankton PCR primers

Type of primer	Primer Name	Sequence
Forward	108F	*5'*- ACAGWAAGACCCTATGAAGCTT- *3'*
Reverse	108R	*5'*- CCTGTTATCCCTAGAGTAACTT - *3'*

Before starting PCR

For phytoplankton metabarcoding, the same strategy (with two PCRs) used for diatoms is recommended. First PCR should be done in the laboratory, and the second one should be performed by the sequencing platform. This means that the abovementioned primers also present the Illumina adapters* for completing the second PCR.

Also, primers (forward and reverse) are usually sold individually at a concentration of 100 µM. Thus, to prepare the primers that will be used for the PCR, we need to dilute them at 1/100 to have a concentration of 1 µM required for the PCR.

Finally, all the following steps need to be done with the maximum precautions under a captair hood, with sterile microtubes and molecular grade water to avoid contamination**.

Example for ~50 samples for phytoplankton:

- Dilute 3 µL of primer (F or R) at 100 µM in 27 µL of molecular grade water
- Dilute 25 µL of this solution into 225 µL of molecular grade water

*/!\ These Illumina adapters may change from one sequencing platform to another.

**/!\ 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)

11.2 Phytoplankton PCR – Protocol

This protocol, including the optimisation of the thermocycler program (Table 4), was developed by Alexis Canino within the OFB and INRAE project PhytoDOM.

Before PCR, dilute your sample DNA to 5 ng/µL concentration.

Material and captair hood preparation

- Clean the station using ethanol or DNA away
- Run UV lights for 15 minutes
- Put PCR primers and KAPA HiFi mix at 4°C
- Take the number of PCR strips you will need for the analysis (one well for each sample, one well for a positive control, and one well for a negative control) and label them
- Take 1 microtube of 2 mL for the PCR mix preparation.

PCR mix preparation

It is important to stress that DNA polymerase present in the KAPA HiFi mix is fragile and all 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).

Table 3. Recommended volume of DNA polymerase and primers for the PCR reaction mix

Reagent	For 1 PCR	For 10 PCR*
KAPA HiFi mix	12.5 µL	137.5 µL
Primer F (1 µM)	5 µL	55 µL
Primer R (1 µM)	5 µL	55 µL

*In reality, it is 11 PCR to account for pipetting errors

How to prepare a reaction mixture step-by-step?

- 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
- Put 22.5 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 2.5 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
- On the thermocycler adjust the program according to the table 4

PCR machine pre-programmed steps

This program was optimized under PhytoDOM OFB – INRAE project.

Table 4. PCR profile adjusted for 108F and 108R phytoplankton primers

Step	Temp (°C)	Time	Cycles
Initialization	95	3 min	
Denaturing	95	30 s	30
Annealing	58	30 s	
Extension	72	30 s	
Final extension	72	5 min	
End	10	∞	

11.3 PCR revelation

Gel preparation

Prepare the gel according to the table below.

Table 5. Amount of reagents and microwave time for a gel preparation.

Size of gel	Agarose (high purity)	TAE Buffer (1%)	Microwave (time)	HD Green Plus DNA Stain*
Small	0.6 g	~ 50 mL	1.30 min	3 µL
Big	1.8 g	~ 150 mL	2 min	9 µL

*Stored at +4°C

How to prepare a gel step-by-step?

- Weigh the required amount of agarose;
- Measure the required volume of TAE buffer;
- Pour TAE into the Erlenmeyer flask with agarose and mix both;
- Heat the solution in the microwave for 1.30 min by taking it out for a short cooling every 30 s;
- After the solution is cooled down enough to be held in the hand, add 3 µL of HD Green Plus and shake the solution gently;
- Place comb(s) in the gel mold to create wells for samples;
- Pour the solution into the gel mold, try to avoid making bubbles;
- Leave the gel to cool down and become solid for approximately. 20 min

Gel loading and migration

- Place the gel into a gel electrophoresis tank (be sure it is completely immersed; otherwise, add more buffer).
- Load 3 µL of PCR amplicon into the gel using loading buffer.
- Load 3 µL of DNA ladder
- Launch migration at 70V, 90 amperes for 40 min (small chamber) or 165V, 200 amperes for 45 min (big chamber).

Gel revelation

- Take the gel from the electrophoresis tank.
- Put it in the gel reader.
- With « preview » mode, verify if it is placed under the camera.
- Launch UV, stop when the image intensity is correct.
- Register the picture.
- Put the gel in the specific container at the end.

12. References

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13. Appendix - Diatom DNA extraction – Summary

1. Sample preparation	Done
Transfert **2 mL** of biofilm in an Eppendorf tube (2mL)	
Centrifuge **30 min** at **18,000 g**	
Remove the supernatant using a pipette	
2. Sample lysis	
Add **700 µL** of **SL1** to the pellet. Homogenize.	
Transfer the solution (biofilm + SL1) into a Nucleospin Bead tube A	
Add **150 µL** of solution **SX**	
Vortex horizontally 5 min at maximum speed	
3. Precipitate contaminants	
Centrifuge the Nucleospin Bead tube A at 11,000 g for 2 min	
Add **150 µL** of **SL3**	
Vortex briefly and incubate at 4°C for 5 min	
Centrifuge at 11,000 g for 1 min	
4. Remove contaminants	
Place the Nucleospin inhibitor removal column (red ring) into a 2 mL tube	
Load 650 µL of clear supernatant	
Centrifuge at 11,000 g for 1 min	
Collect the filtered liquid in a clean 2 mL tube	

Repeat until there is no more supernatant	
Discard the Nucleospin inhibitor removal column	
5. Adjust binding conditions	
Add 250 µL of SB buffer	
Vortex briefly and do a spin-down	
6. Bind DNA	
Place a Nucleospin Soil column (green ring) into a 2 mL tube	
Load 650 µL of the sample in the column	
Centrifuge at 11,000 g for 1 min	
Discard the flow-through and put the column back into the tube	
Repeat these steps until there is no more sample	
7. Wash DNA	
Add 500 µL of buffer SB into the Nucleospin Soil column	
Centrifuge at 11,000 g for 30s	
Discard the flow-through and put the column back into the tube	
Add 550 µL of SW1 into the Nucleospin Soil column	
Centrifuge at 11,000 g for 30s	
Discard the flow-through and put the column back into the tube	
Add 650 µL of SW2 into the Nucleospin Soil column	
Centrifuge at 11,000 g for 30s	

Discard the flow-through and put the column back into the tube	
Add 650 µL of SW2 into the Nucleospin Soil column	
Centrifuge at 11,000 g for 30s	
Discard the flow-through and put the column back into the tube	
8. Dry silica membrane	
Centrifuge at 11,000 g for 2 min	
9. Elute DNA	
Place the Nucleospin Soil column into a new 1.5 mL tube	
Add 30 µL of SE solution	
Incubate at room temperature for 1:30 min (do not close the lid)	
Centrifuge at 11,000 g for 30s	
Discard the Nucleospin soil column and keep the tube containing the DNA	
Store the tube at -20°C	

14. Appendix - Phytoplankton DNA extraction - Summary

1. Sample preparation	Done
Let the Sterivex (if frozen) at 4°C for 30 min	
Vortex horizontally 5 min at maximum speed	
2. Sample lysis	
Add 40 µL of lysosyme into the Sterivex and incubate at 37°C for 45 min	
Add 50 µL of proteinase K and 100 µL of SDS - incubate at 55°C for 2 h	
3. Precipitate contaminants	
Aspirate the entire volume of the Sterivex and put it into two 2 mL tube	
Add 200 µL of SL3 in both tubes	
Vortex briefly and incubate at 4°C for 5 min	
Centrifuge at 11,000 g for 2 min	
4. Remove contaminants	
Place the Nucleospin inhibitor removal column (red ring) into a 2 mL tube	
Load 650 µL of clear supernatant	
Centrifuge at 11,000 g for 1 min	
Remove the column and close the tube containing the flow-through	
Repeat until there is no more supernatant; Each time, collect DNA in a clean tube	
Discard the Nucleospin inhibitor removal column	
5. Adjust binding conditions	
Add 250 µL of SB buffer to each tube	
Vortex briefly and do a spin down	
6. Bind DNA	**Done**
Place a Nucleospin Soil column (green ring) into a 2 mL tube	
Load 650 µL of sample in the column	
Centrifuge at 11,000 g for 1 min	
Discard the flow-through and put the column back into the tube	
Repeat these steps until there is no more sample	
7. Wash DNA	

Add 500 µL of buffer SB into the Nucleospin Soil column	
Centrifuge at 11,000 g for 30 s	
Discard the flow-through and put the column back into the tube	
Add 550 µL of SW1 into the Nucleospin Soil column	
Centrifuge at 11,000 g for 30 s	
Discard the flow-through and put the column back into the tube	
Add 650 µL of SW2 into the Nucleospin Soil column	
Centrifuge at 11,000 g for 30 s	
Discard the flow-through and put the column back into the tube	
Add 650 µL of SW2 into the Nucleospin Soil column	
Centrifuge at 11,000 g for 30 s	
Discard the flow-through and put the column back into the tube	
8. Dry silica membrane	
Centrifuge at 11 000 g for 2 min	
9. Elute DNA	
Place the Nucleospin Soil column into a 1.5 mL tube	
Add 30 µL of SE solution	
Incubate at room temperature for 1 min 30 s (do not close the lid)	
Centrifuge at 11,000 g for 30 s	
Discard the Nucleospin soil column and keep the tube containing the DNA	
Store the tube at -20°C	