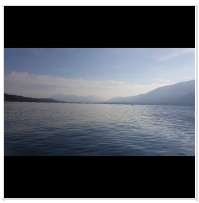

Feb 10, 2021

updated version- Lake biofilms sampling for both downstream DNA analysis and microscopic counts

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EcoALpsWater

Marine Vautier

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The objective of this protocol is to provide a reliable and replicable method for the sampling of lake micro-phytobenthos and associated microbes in biofilms, to be used in both downstream DNA analysis and algal microscopic counts.
The field protocol is optimized for routine sampling and is in agreement with CEN guidance (NF EN 13946) and CEN technical report (CEN/TR 17245) for the analysis of benthic diatoms from rivers and lakes. The application proposed here in the context of EcoAlpsWater aims at comparing DNA inventories to traditional inventories (microscopy).
The sampling strategy has been designed to assess localized hotspots pollutions/perturbations impacts on the shoreline, taking into account shoreline's heterogeneity.
This protocol is part of the deliverables provided by the WP1 of the Eco-AlpsWater project. All members of the EcoAlpsWater consortium have contributed to the optimization of this protocol.
The use of microalgae to assess ecological quality of lakes is traditionally based on phytoplankton that allow to evaluate the trophic status. A single depth-integrated water sample from one sampling site above the deepest point of the lake is chosen, and samplings are carried out in the euphotic zone at different seasons.
Though not always included as biological quality indicator in lake monitoring, phytobenthos has relevance for lake ecological quality assessment and might be used as a complementary indicator beside phytoplankton in the future (e.g. DeNicola & Kelly 2013).
Phytobenthic communities of the lake's shorelines are generally heterogeneous. The reason for this heterogeneity can be due to many factors, including substrate characteristics as well as anthropogenic factors such as eutrophication, the presence of pollution hotspots, like arrivals of polluted rivers in the lakes or arrivals of wastewaters through drains (e.g. Crossetti et al. 2013, Rimet et al. 2015/2016/2018, Cicek & Yamuc, 2017). But it can also be caused by the nature of the neighboring terrestrial habitats and land use (for instance closed forest vs open fields, or pasture vs steep rock falls).
The objective of this protocol is to provide a reliable and replicable method for the sampling of lake micro-phytobenthos and associated microbes biofilms to be used in both downstream DNA analysis and algal microscopic counts. Examples of applications to assess localized hotspots pollutions impact on the shoreline, taking into account shoreline's heterogeneity, will be provided.
The field protocol is based on routine methods used for biofilms sampling and is in agreement with :
- CEN 2014. Water quality - NF EN 13946 - Guidance for the routine sampling and preparation of benthic diatoms from rivers and lakes. Afnor, 1-23.
- CEN 2018. Water quality - CEN/TR 17245 - Technical report for the routine sampling of benthic diatoms from rivers and lakes adapted for metabarcoding analyses. CEN, 1-8.

DOI

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lake, biofilm, diatoms, sampling, biodiversity, DNA analysis, microscopic counts

protocol ,

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- When & where to sample
  - Choice of the sampling season and period
  - Choice of the sampling stations (example of application in Eco-AlpsWater monitoring)
- Biofilms sampling procedure & preservation
  - If stones are available
  - If stones are not available
  - Blank samples
- Label standard and sampling filed datasheet

- Reagents
  - for materials cleaning
    * 10% H_2O_2 solution
    * DNA free water (Millipore Water (Milli-Q) 18.2 MΩcm (at 25 °C)

  - for sampling
    * DNA free water (Millipore Water (Milli-Q) 18.2 MΩcm (at 25 °C), plan 1L for 10 stations
    * for the preservation of DNA samples: absolute ethanol (high quality - for analysis), plan approx. 500mL (for 10 stations)
    * for the preservation of samples dedicated to microscopic counts: absolute ethanol, plan approx. 500mL (for 10 stations) **OR** another solution as formaldehyde (according to the countries the traditional protocol for fixation of samples can differ).
    * for blank sample: DNA free water (Millipore Water (Milli-Q) 18.2 MΩcm (at 25 °C), plan 50mL per blank sample and absolute ethanol (high quality - for analysis), plan approx. 50mL per blank sample

- Materials
  - DNA free tray, 1 per sampling station and 1 per blank sample

- Consumables
  - new nylon brush (e.g. toothbrush), 1 per sampling station (to avoid contaminations)
  - 50mL Falcon tube (sterile) :
    * at least 3 tubes per sampling station:

1 tube for DNA analysis, 1 tube for microscopic counts and 1 tube for additional sample kept without fixation (if some samples have to be inspected live under the microscope, or for cyanotoxins analysis)

    * 1 per blank sample: it is recommended to make a sample blank every 10 samples approximately
  - gloves

- Absolute ethanol
  CAS number : 64-17-5
  Signal word : Harmul and Flammable
  Hazard phrases : 225, 319
  Precaution phrases : 210, 305+351+338

- Formaldehyde solution
  CAS number : 50-00-0
  Signal word : Flammable, Corrosive substance, Toxic, Health hazard
  Hazard phrases : 226, 301+311+331, 314, 317, 335, 341, 350, 370
  Precaution phrases : 201, 210, 280, 301+310+330, 303+361+353, 305+351+338+310

- 10% H_2O_2 solution, Hydrogen peroxide solution at 10%
  CAS number : 7722-84-1
  Signal word : Harmful and Corrosive substance
  Hazard phrases : 302, 318, 412

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Precaution phrases : 273, 280, 301+312+330, 305+351+338+310

- Read and follow the step 1 - When & where to sample
- Watch the sampling desmonstration video: **https://youtu.be/_6Q48nSMjNA**
- The following cleaning precautions must be applied, to avoid contaminations :
  - Nylon brushes (e.g. toothbrush) must be new
  - At the lab, the tray is cleaned with 10% H_2O_2, then rinsed with DNA free water and dried
  - Wear gloves throughout the sampling process and change them between different sampling stations

When & where to sample

1 ▪ **Choice of the sampling season and period**

- Phytobenthic communities' composition are changing along seasons. In large lakes, the major variables explaining these temporal changes are nutrients (and phosphorus especially).

- Moreover, the heterogeneity between the communities present along the shoreline vary from a season to another, and is more important in summer (e.g. Lake Geneva in Rimet et al. 2015). For this reason, **samplings must be carried out during summer**.

- In general sampling sites free of submerge (and emerge) macrophytes should be selected to exclude shadowing effects on phytobenthic growth which is (ironically) most effective during summer.
  - **If wind induced strong waves** during several days and scoured the biofilms (e.g. storms, days with continuous strong winds), then, you have to **wait for 2-3 weeks** before collecting the samples, to allow biofilms to restructure.
- Water level fluctuations would also have a significant effect on biofilms community composition. **Water level should be stabilized at least over the last 3 weeks and the samples should be taken at 50cm depth from minimum water level** (recorded annual data).

▪ **Choice of the sampling stations (example of application in Eco-AlpsWater monitoring)**

▪

In the framework of Eco-AlpsWater, at least **10 stations** must be sampled in each lake. Usually, samples are taken every 0.5 to 1.5 km along the shoreline (depending on lake size). Since 10 samples are a quite reduced number of samples to cover the entire perimeter of a lake, we propose to **choose a small stretch shoreline** of the lake. This stretch should be chosen by expert judgment and **should present as small heterogeneity in substrate**.

Sampling site selection strategy (figure 1):
- When shoreline is homogeneous (when natural, or semi-natural), position one sampling site every 0.5 to 1.5 km (depending on lake size).
  - When heterogeneity is present along the shoreline, densify the number ofsampling sites, for instance:
    * near the arrival of a river (e.g. in river mouth, and one on each bank)
    * near the arrival of a wastewater treatment plant

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* inside/outside harbors (inside=in the harbor, outside=in the lake)
* near quarry exploitation (aggregate, stones, sand exploitation)

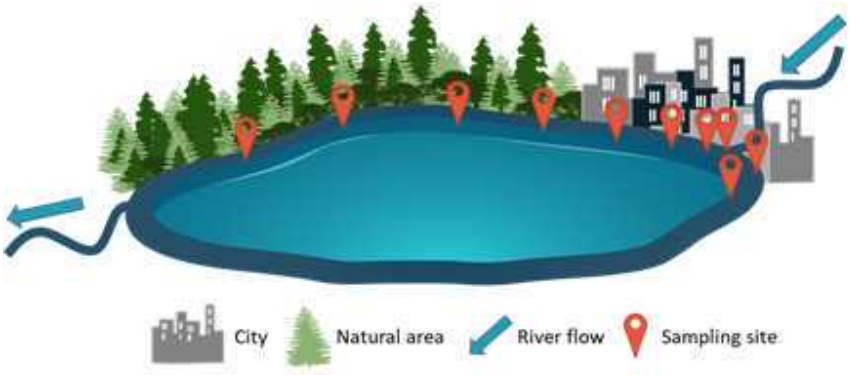

Figure 1: Example of positioning of the 10 sampling stations along the shoreline of a lake

Biofilms sampling procedure & preservation

2 ▪ The following cleaning precautions must be applied, to avoid contaminations:
- Nylon brushes (e.g. toothbrush) must be new
- At the lab, the tray is cleaned with 10% H_2O_2, then rinsed with DNA free water and dried
- Wear gloves throughout the sampling process and change them between different sampling stations

2.1 **If stones are available** (figure 2):
A demonstration video is available at https://youtu.be/_6Q48nSMjNA

▪ Take at least 5 stones (it can be more, depending on stones sizes and biofilms amounts), for a total brushed surface of at least 100 cm^2. Stones are taken at 20-50 cm depth from the minimal water level (annual data) in an area of 100 m². The area is at least 2 m wide (a 2 m wide strip corresponds to a 50 m long stretch can be sampled).
▪ Let the stones drain for a few minutes
▪ Fill the bottom of the tray with 50 ml of free DNA water
▪ Brush the stones in the tray.

Note: Brush the stones above and on the sides, but not the surface in contact with the ground.

▪ The obtained biological material (mixture of biofilms and water) is then sub-sampled:

* For DNA samples (figure 2):
- Take the biofilm/water mixture from the tray and fill the tube up to 10 ml
- Complete the tube with absolute ethanol up to 50 ml (add ~40 mL of absolute ethanol)
- Shake to homogenize, label
- The samples are stored at 4°C in the dark for a maximum of 1 month, or can

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also be frozen at -20 or -80°C for a maximum of 3 months

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

* For microscopy samples:

- Take a second sub sample (10 mL) of the biofilm/water mixture from the same tray
- Add the appropriate preservative solution for the microscopy analysis according to the case, preservative solution can be Ethanol (70%) or Formaldehyde (2%).Store at ~ +4°C, in the dark.

- If the sample has to be be inspected live under the microscope (in particular for soft algae), a third sub-sample can be kept without any fixation. Store at ~ +4°C, in the dark.

2.2

If stones are not available:

- Sample on artificial and hard substrates (figure 3) (e.g. riprap, artificial concrete banks)
- Use a hoe equipped with a net
- Scrap a minimal surface of 100 cm^3 at 20-40 cm depth

Figure 3: Schematic sampling procedure in the field when no stone is available

- If artificial and hard substratesare not available, sample on macrophytes

- At 20-40 cm depth: squeeze submerged filaments (e.g. Elodea, Potamogeton) or scrap the macrophyte stem (e.g. Typha). At least 20 cm of stem must be

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scrapped.

Important note: if samples are taken on macrophytes, this must be indicated on the sample, and only diatom analyses will be performed on these samples.

Blank sample

3 It is recommended to make a blank sample during the sampling for DNA analysis:
- If number of stations < 10, make 1 blank sample
- If number of stations > 10, make 1 blank sample every 10 samples approximately

- Fill the bottom of a clean tray with 50 ml of free DNA water.
- Fill a 50 mL sterile Falcon tube up to 10 ml with the water from the tray.
- Complete the tube with absolute ethanol up to 50 ml (add ~40 mL of absolute ethanol).
- Shake to homogenize and label.
- Store the blanks like the other samples (at ~ +4°C in the dark for a maximum of 1 month, or frozen at -20 or -80°C for a maximum of 3 months).

Label standard & sampling field datasheet

4
- Sample labeling: “Biofilm”, “River Name”, “Station”, “Date”
- Accompanying documents:

- field sheets : we propose a field sheet (Table 1) for mesological accompanying data with four subparts (Figure 4):
    * A: Station description
    * B: Sample description
    * C: Shore description
    * D: Land use description
- photographic documentation : if possible, add a photographic documentation of the sampling area

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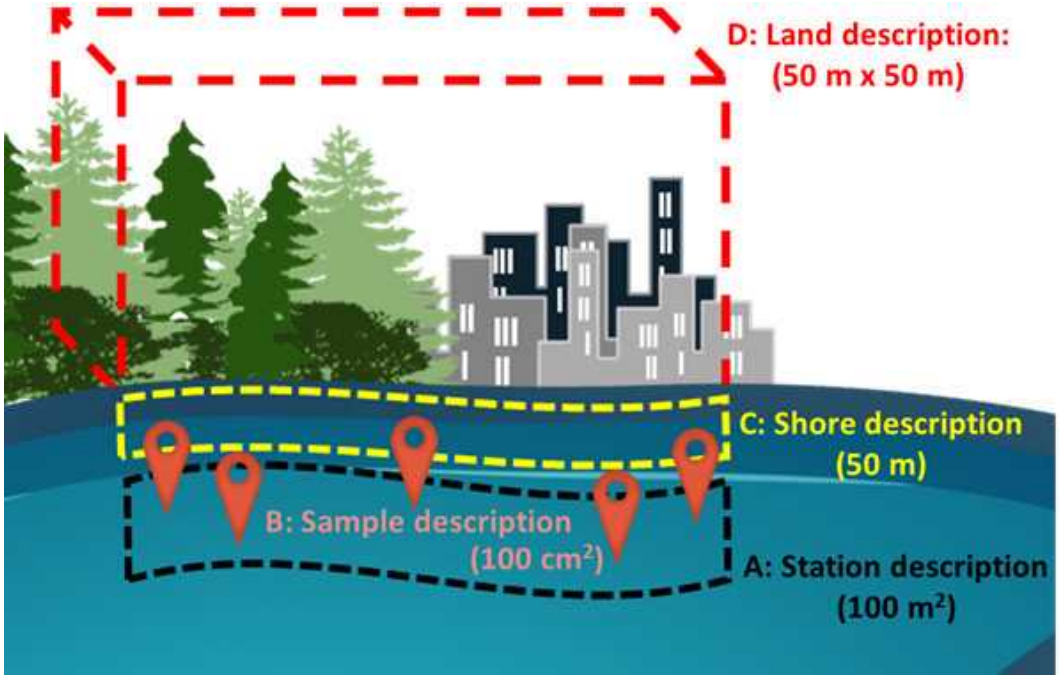

Figure 4: Presentation of the description sheet for one station

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BIOFILM sampling - Lakes			ECO-ALPSWATER INTERREG
(Fields marked with * are required)			
Name of the lake *			
Sampling person(s) *			
Sampling date and time *			
A: STATION DESCRIPTION	Area in which the stones are taken (100 m^2)		
Name of the sampling Station *			
Coordinates *			
Nearest place, municipality			
Waves (tick)*	no	a little	a lot
Weather condition (tick)*	Nice	Mixed	Bad
Wind (tick)*	No	Weak	Strong
Current wind direction *			
Current wind speed *			
Wind direction (last 2 weeks)			
Wind speed (last 2 weeks)			
Shading (tick)*	Open	semi-open	Closed
Shading of survey site [%] *			
Water level (as referred to annual minimum water level)			
Possible pollution sources (eutrophication)			
Station average depth*			
Average slope (%)*		**Distance (cm)**	
		Depth (cm)	
Water Physico-chemistry			
pH			
Dissolved oxygen (mg.L-1)			
Dissolved oxygen (%)			
Conductivity (µS.cm-1)			
Temperature (°C)			
turbidity			
PAR / light			
Chl a (µg/L)			
Biofilm dry weight		**Surface (cm^2)**	

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Hydrology*	Code	Tick	Percentage (%)
River coming			
River outing			
Substrate *	*2 substrates to tick, 1 dominant + a 2nd*		
Vase (<2µm)			
Sand, silt (2µm-2mm)			
Gravels (2mm-2cm)			
Stones (2cm-20cm)			
Blocks (>20cm)			
Flagstone			
Vegetation			
Helophyte			
Overhanging vegetation			
Alive emerging ligneous vegetation			
Dead ligneous vegetation			
Hairy root			
litter or coarse organic debris			
bryophytes			
Floating hydrophytes			
Submerged hydrophytes			
Additional Notes			

B: SAMPLE DESCRIPTION	Substrate on which the biofilm is sampled (100 cm^2)		
Sampling depth (cm)*			
Sampling substrate*	Code	Tick	Percentage (%)
Gravels (2mm-2cm)			
Stones (2cm-20cm)			
Blocks (>20cm)			
Additional Notes			

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C: SHORE DESCRIPTION	Shoreline bordering the sampled station (50 m)		
Shore composition	Code	Tick	Percentage (%)
Rockfill / riprap			
Sediments / clays			
Sand			
Gravel / Stones			
Cliff			
Blocks			
Bridge			
Dam / Wall			
Harbor			
Pier / Dock			
Launching / Wedge			
Duckboard			
Additional Notes			

D: LAND DESCRIPTION	Land bordering the sampled station (50 m x 50 m)		
Land composition	Code	Tick	Percentage (%)
Bridge			
Harbor			
Pier			
Dock			
Track / Road			
City / Housing			
Forest			
Beach			
Forest			
Pond / Watercourse			
Reeds			
Parks / gardens			
Agricultural fields / Vineyard			
Additional Notes			

Table 1: Field datasheets in 4 parts (A, B, C, D)

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