Eco-AlpsWater

Innovative Ecological Assessment and Water Management Strategy for the Protection of Ecosystem Services in Alpine Lakes and Rivers

Priority 3: Liveable Alpine Space. SO3.2 - Enhance the protection, the conservation and the ecological connectivity of Alpine Space

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Lake and river eDNA Fish sample collection from the field for downstream molecular analysis

Interreg Alpine Space - Eco-AlpsWater project – WP1

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I. ABSTRACT

The objective of this protocol is to provide a reliable and replicable method for the sampling of lake and river fish to be used for downstream DNA analysis. This protocol is one of those proposed by the Eco-AlpsWater consortium to promote the implementation of High Throughput Sequencing (HTS) of environmental DNA (eDNA) in the biomonitoring and ecological assessment of water bodies in the European Alpine region. The application proposed here, in the context of Eco-AlpsWater, aims at comparing DNA inventories to traditional fish inventories. The sampling design varies between lakes and rivers. This protocol is part of the deliverables provided by the WP1 of the Eco-AlpsWater project. All members of the Eco-AlpsWater consortium (involving 12 partners: http://www.alpine-space.eu/projects/eco-alpswater/en/home) have contributed to the protocol optimization.

II. GENERAL DESCRIPTION OF SAMPLING IN LAKES AND RIVERS

The general sampling protocol to be implemented for the Eco-AlpsWater project consists of an integrated sampling workflow. This strategy is mainly based on two principles: i) the collection of a large volume (*ca*. 30 L) of water along the lakes shoreline or within the main river flow to be representative of the waterbody and to increase the chance of collecting rare DNA, and ii) a filtration in a closed cartridge to capture the eDNA and to limit potential contamination. The sampling design varies between lakes and rivers and is described in detail below. The amount of suspended matter could cause the blocking of the filter cartridge before the required 30 L of water have been processed. Depending of the charge of suspended matter two strategies can be adopted: i) if the charge is low, the water can be pumped and filtered directly, or ii) if the charge is high and the risk of clogging too high, the 30 L of water could first be collected in a clean DNA-free recipient on the boat. After the full samples have been collected the water will be homogenized and then the maximum amount of water is filtered before the clogging of the filter occurs. In the latter case, one clean DNA-free recipient should be used per filtration to limit potential cross-contamination.

During the preparation of the sampling protocols there have been numerous discussions on whether or not other approaches (i.e. point sampling) would not be more suitable. To address this uncertainty a point sampling approach has been implemented alongside the integrated approach in 4 key lakes (i.e. Lake Bourget, Lake Garda, Lake Mondsee and Lake Starnberg; see page 18 and 22).

Sampling for fish eDNA should ideally be carried out before the traditional fish monitoring methods are deployed. The reason for this is that sampling gear for traditional fish monitoring is often used at multiple locations and can thus transfer fish DNA between water bodies. Collecting eDNA samples one week before starting the traditional surveys would thus ensure that no 'foreign' fish DNA will be detected and only the local fish biodiversity willbe assessed. If sampling before the traditional surveys is not possible it is recommended to conduct the eDNA sampling two weeks after the traditional surveys. **Most importantly, in no circumstances should eDNA samples be collected at the same time as traditional fishing gear is deployed or retrieved!** Finally, for similar reasons the collection of eDNA samples should be performed by a different boat then the one used for traditional monitoring surveys. However, if this is not possible contamination can be avoided by only collecting samples from the bow of the boat (i.e. the front of the boat).

NOTE: Each of the following approaches has its advantages and disadvantages and are to be considered as still in development. The VigiDNA® sampling method proposed here, was implemented as main part of the fish eDNA sampling in the Eco Alps-Water project. Although the approach is user-friendly and time-efficient, this method cannot be further recommended due to several reasons: (i) the method was not reproducible among all project partners (ii) improper storage and bacterial growth in the storage buffer led to DNA degradation and reduced DNA extraction efficiency (iii) the filter and buffer solutions used are no longer sold individually, but only in combination with extraction and analysis done by the vendor. The other methods proposed here (Sterivex & GFC point sampling – see appendix) were not applied by all project partners, but for those who did use them, they worked very well.

II.1 Lake sampling design and effort

Key Lakes

The sampling effort for the key lakes should consist of multiple samples collected along the shoreline of the lake and at least one sample being collected from the different depth strata (i.e. from 0 m depth to 1-2 m above the bottom). Ideally the sampling effort is determined by the lake size but each PP will need to evaluate their budget to determine the most suitable sampling effort. A proposed sampling effort for the key lakes can be found in Table 1. However, the final sampling effort used will need to be determined by each PP individually.

Table 1. Proposed sampling effort for the key lakes within the Eco-AlpsWater project.

Surface area (km^2)	No. of shoreline samples	No. of depth samples	Lake name	Shoreline length (km)	Lake depth (m)
< 5	2	1	Bled	5.6	30.6
5-20	4	1	Mondsee	25	68
21-60	6	1	Lugano	85	28
			Starnberg	45	128
			Bourget	53	145
> 60	6	1	Garda	370	346

Sampling of the shoreline

Shoreline samples should be collected from the boat's bow along linear transects(s) as close as possible from the shoreline, at least in the area between 0 to 20 m from the shore. A starting point has to be chosen and geolocated (for instance the northern part of the lake) from which the boat will follow the shoreline either in the clockwise or counter clockwise direction but always moving in the same direction (without going back). At a minimum geographic coordinates of the transect should be recorded at the start, middle and end of the sampling transect. However, it is highly recommended that PPs record the coordinates ofthe entire sampling transect through a GPS or a smartphone (e.g. using OruxMaps application) such as the coordinates of the entire tracks could be downloaded later. Boat perturbations should be avoided or reduced at their minimum. Thirty litres of water has tobe pumped with a constant flow (ideally 0.5 L/min), at a constant depth and near the surface(e.g. 15 cm). Pumping of the water can be done using a peristaltic pump with a flow of ca.
0.5 L/min. The easiest and most direct approach of collecting the samples is to combine the collection of the water and filtration into one single step by connecting the pumping system with the cross flow filtration capsule (i.e. VigiDNA® 0.45 µm capsule or any similar cartridge with a pore size of 0.45 µm; Figure 1). Alternatively, water samples can be collected along the transect and pumped into a clean DNA-free recipient on the boat, homogenized and then filtered. In the latter case, a different recipient should be used for the different transects to avoid cross-contamination between samples.

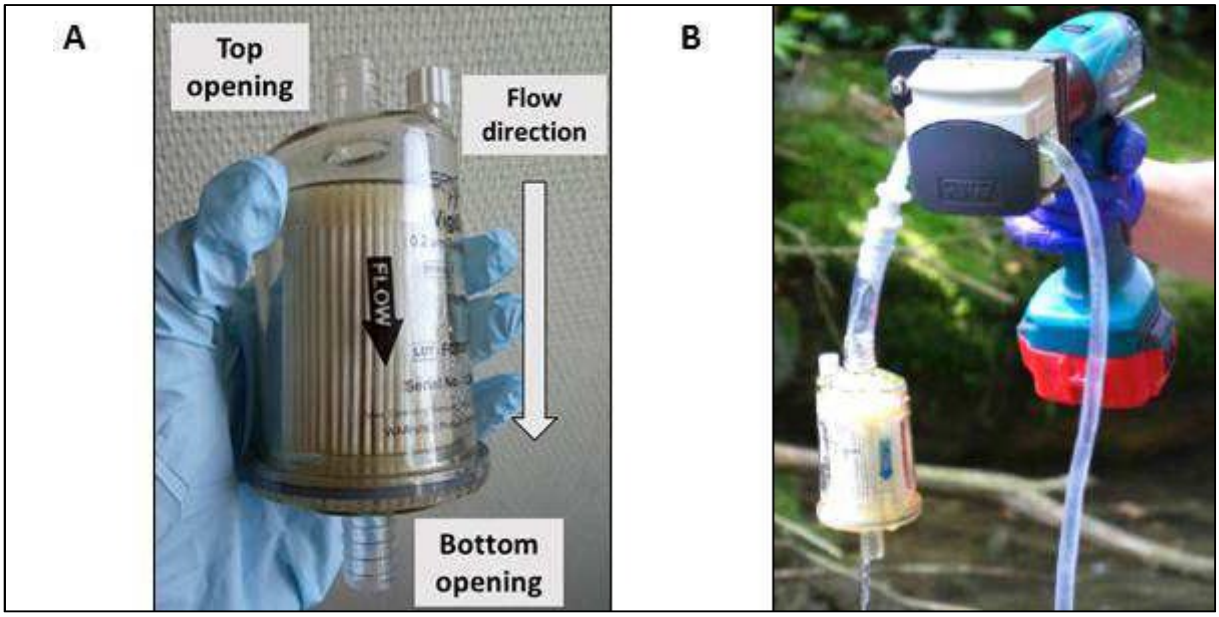

Figure 1. The cross flow filtration capsule (pore size of 0.45 µm) designed for filtering large water volumes (i.e. VigiDNA®) (A) and the set-up that can be used to combine the collection and filtering of the eDNA samples for fish (B).

A single sampling unit thus consists of a 30 L water sample filtered through a single cartridge. A sampling transect should be maximum 6 km long (or less if the total shoreline length is < 6 km). For large lakes a maximum of 6 cartridges are to be collected. This means that 6 linear transects (max 6 km each) are to be selected. These 6 transects can be either spatially distributed around the lake, or chosen according to habitats heterogeneity. **In all circumstances, it is highly recommended to avoid area where tributaries flow into the lake.**

Sampling of the depth strata

The collection of the depth sample should be performed at three different stations, spaced evenly along the main axes of the lake, in the deepest zone of the lake. Samples should be collected from the different depth strata (i.e. from 0 m depth to ca. 1-2 m above the bottom). In all circumstances disturbing the bottom substrate during the collection of samples should be avoided. At each sampling location, a 12 L depth integrated water sample should be collected using either an Integrated Water Sampler (e.g. Hydrobios IWS) or a pool of discrete samples collected with a Niskin bottle. In the latter case, 2 L of water could be collected at 6 equal depth intervals (e.g. for a station with a maximum depth of 115 m samples could be collected at 0, 20, 40, 60, 80 and 100 m depth) and combined into a clean DNA-free recipient (e.g. carboys or bottles). When moving to the next sampling station it is recommended to store the collected water cooled (e.g. using an ice bath) to avoid degradation of the eDNA. Once all three 12 L depth integrated water samples have been collected and combined, homogenize the 36 L sample and proceed immediately with filtering a volume of 30 L using the cross flow filtration capsule.

Additional Lakes

The sampling effort and design for the additional lakes will need to be decided on by the individual PPs individually depending on the available budget. Here, some general guidelines are given for the sampling of additional lakes.

For additional lakes, a simplified protocol using a single cross flow filtration capsule (i.e. VigiDNA® 0.45 µm capsule) is proposed. For each additional lake 2 L subsurface watersamples (i.e. 10-20 cm below the water surface) should be collected 16 times from the littoral zone. One clean DNA-free sampling bottle could be used to collect all samples from a single lake as all samples from a single lake will eventually be combined into a larger clean DNA-free recipient (capacity > 30 L). When collecting samples, special care should be takento collect samples representative of the entire lake and from all the different habitat types occurring at the littoral zone (i.e. ensure a proper spacing of all collected samples). Disturbing the bottom substrate should be minimized during sample collection (i.e. this is especially important when samples will be collected by wading into the lake). When all samples have been collected and combined, homogenize the water collected and proceedby filtering 30 L of water with the cross flow filtration capsule as described previously.

If sufficient funds and time are available a depth integrated sample could also be collected for the additional lakes. The suggested protocol will follow that of the depth integrated samples for the key lakes.

II.2 River sampling design and effort

Key Rivers

For each key river a number of sampling stations need to be selected by each PP taking into consideration i) the length and heterogeneity of the river and ii) the available budget. If multiple station will be sampled for a given river, sampling stations should be separated by several kilometres (i.e. ≥ 10 km) to ensure they can be treated as truly independent sampling sites (i.e. eDNA in rivers can be transported over several kilometres). At each station two sampling replicates should be collected. A single sampling replicate will consist of collecting and filtering a large volume of water (30 L) from a single sampling point within the main stream channel in the area of fastest flow. A similar approach to the integrated samples from the key lakes can be used to simultaneously collect and filter the water samples (i.e. using a peristaltic pump to directly collect and filter water through the cross flow filtration capsule using a flow of 0.5 L/min . Alternatively, a number of discrete samples could be collected from the area of fastest flow (e.g. six 5 L samples collected using a clean DNA-free bucket over 30 min time span) and combined into a large clean and DNA-free recipient (capacity > 30 L). When all discrete samples have been collected homogenize the sample andproceed by filtering 30 L of water through the cross flow filtration capsule.

Additional Rivers

The sampling protocol for the additional rivers will be identical to the one described for the key rivers with the only exception that only one 30 L sample needs to be collected per sampling station. As with the protocol for the key rivers, all PPs need to determine the number of sampling stations based on i) the length and heterogeneity of the river and ii) the available budget.

II.3 Filtration, sample preservation and storage

After all the water volume has been filtered through the VigiDNA® filter capsule ensure that excess water is removed and close off the outlet of the filter capsule. To preserve the eDNA, add 80 mL of preservation buffer (Tris–HCl 0.1 M, EDTA 0.1 M, NaCl 0.01 M and N-lauroyl sarcosine 1% with pH 7.5–8) through the inlet and outlet of the capsule and close off the inlet and outlet properly. Place the cartridge horizontally and shake it from left to right for 1 minute. Label both the filter capsule and a sterile bag with the standard labelling format given in Table 2. Place the filter capsule in the sterile bag and place both capsule and bag back into the initial storage box of the filter capsule. Store samples in a cooling box while ensuring that the cartridges are kept in a vertical position and stored in the dark at room temperature up to 3 months. **In no circumstance should the cartridge be stored below 0 °C.**

Figure 2. The preservation buffer can be added to the VigiDNA® filter capsule using a small DNA-free funnel. Important: ensure that the outlet of the filter is closed before adding the preservation buffer!!!

Table 2. Standard format to be used for the labelling of fish eDNA samples.

Water body	Sampling approach	Sampling location	Label
Lake	Integrated	Shoreline	"Fish", "Lake name", "Station (1)", "Date", "Volume (mL)"
		Depth	"Fish", "Lake name", "Stations (3)", "Date", "Depth range (min-max) (m)", "Volume (mL)"
	Point	Shoreline	"Fish", "Lake name", "Station (1)", "Date", "Volume (mL)"
		Depth	"Fish", "Lake name", "Station (1)", "Date", "Depth range (min-max) (m)", "Volume (mL)"
River	Integrated	Mid channel	"Fish", "River name", "Station (1)", "Date", "Volume (mL)", "Replicate No."

III. MATERIALS CHECK LIST AND PRECAUTION DURING SAMPLING

III.1 Materials check list

- Peristaltic pump (the recommended model is the Vampire Sampler [Burkle] although other peristaltic pumps which allow regulating the speed of pumping can be used).
- Sampling pole (or stick) and adhesive tape (used to fix the tubing and the strainer to the pole and hold them below the water surface during sample collection).
- Tubing (one silicone tube of about 1-1.5 m long, wall thickness = 1.6 mm, internal diameter = 8.0 mm, outside diameter = 11.2 mm).
- Clean DNA-free sampler (e.g. Niskin bottle, plankton sampler or integrated sampler) for the collection of samples from the different depth strata (only needed for key lakes, 1 required for each sampling campaign).
- Large recipient (capacity > 30 L) with volumetric indicator marks.

  NOTE: Depending on the sampled water body and the approach used for collection of the water samples the number of recipients will differ. For example, for the collection and direct filtering of integrated samples from the lakes one recipient is sufficient. However, for the integrated samples from the key rivers which will be collected through taking and combining multiple discrete samples a minimum of two clean and DNA-free recipients are needed.

- Single use sampling kits for the collection and processing of transect samples (one kit for each transect). We recommend PPs to purchase the complete sampling kits from SPYGEN (cost: 66 EUR) which include all materials needed except for the tubing (extra cost: 18 EUR, page 15 for full details). Alternatively, sampling kits could be assembled by the PPs for which they need the following items:
    - Clean DNA-free zip lock bag to store all materials before collecting samples (1 bag containing all required materials for the collection of a single sample).
    - VigiDNA® filter capsules to be purchased from SPYGEN (cost: 60 EUR).
    - Tubing (two silicone tubes of about 1-1.5 m long, wall thickness = 1.6 mm, internal diameter = 8.0 mm, outside diameter = 11.2 mm).

      NOTE: Depending on the peristaltic pump used the optimal tube specifications may differ but the tubing should fit both the pump and the cross flow filter cartridge.
    - Strainer adapted to tube diameter (to avoid the collection of large particles which may block the filter capsule).
    - Preservation buffer (Tris–HCl 0.1 M, EDTA 0.1 M, NaCl 0.01 M and N-lauroyl sarcosine 1% with pH 7.5–8) prepared at the lab or the buffer provided with the VigiDNA® 0.45-µm capsule (SPYGEN). Approximately 100 mL is needed per filter capsule (i.e. ca. 80 mL to fill the capsule and 20 mL of excess buffer in case it is needed).
    - A small DNA-free funnel to fill the cartridge with buffer (1 for each sample).
    - Labelled sterile bags to store the cartridge after filtering (1 for each sample).
    - Gloves (a minimum of 3 pairs per sample).

  Depending on your sampling setup, the tube length that come with the Spygen Kit may not be sufficient. It is advised to purchase additional tubes and tube connectors. Other than the tubes included in the Spygen Kit, these (additional) tubes need to be cleaned (10% H_2O_2) prior to use.

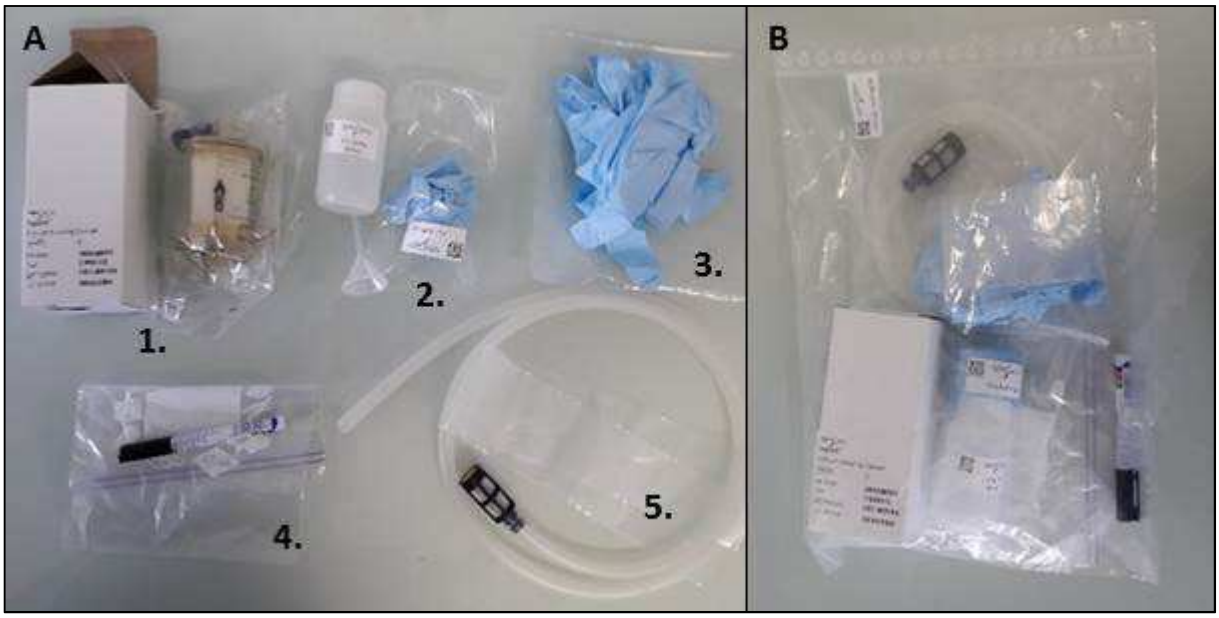

Figure 3. Overview of the equipment needed for sample collections. In panel A from left to right and top to bottom: 1. Cross flow filter cartridge and caps; 2. Preservation buffer and funnel; 3. Gloves; 4. Clean bag and permanent marker; 5. Tubing and strainer. In panel B a single use sampling kit containing all sampling equipment in clean bag is shown.

- Filtering equipment needed to collect a blank equipment control before each sampling campaign (see page 11 for full details):
  - 1 L of DNA free water (e.g. Milli-Q water).
  - Sterile syringe with 60/100 mL capacity (1 is needed for every campaign).
  - Sterile Sterivex filtering unit with a pore size of 0.45 µm (1 for each campaign) and caps to close off the inlet and outlet (Luer-lock male and female).
  - 1000 µL pipette and sterile 1000 µL filter tips.
  - Preservation buffer (3 mL for each campaign).
  - Parafilm (optional but recommended).
  - Gloves (a minimum of 2 pairs per sample).
  - Labelled sterile bags (or 50 mL falcon tubes) to store the cartridge after filtering (1 for each sample).

III.2 Precautions during sampling

- Store all materials inside clean bags or containers to reduce any contamination.
- Wear gloves during the whole procedure and do not let gloves contact contaminated surfaces, such as any equipment that cannot be sterilized.
- Prior to sampling, the bottles/containers and all materials that will come into contact with the water samples will need to be cleaned to remove potential contaminant DNA. All equipment should be washed with a detergent and thoroughly rinsed with tap water. Subsequently, contaminant DNA needs to be removed by washing equipment with a 10% H_2O_2 or a 20% commercial bleach solution (i.e. ca. 1.2%available chlorine). To ensure that all DNA is removed it is recommended that all equipment is exposed to the cleaning agents (H_2O_2 or bleach) for 10-20 min. Finally, rinse all equipment three times with distilled or Milli-Q water before letting it air dry (preferable not in a laboratory environment where DNA is being handled).
- At the sampling site, rinse sampling materials (e.g. Niskin bottles, integrated sampler and/or sampling bottles) three times using sampling water to remove any remaining bleach before collecting sample.
- During the handling and storage of water samples try to reduce exposure to sunlight if possible. Also, when water samples will not be filtered immediately it is highly recommended to store samples on ice to avoid the degradation of eDNA.
- If the strainer is not attached to the tube, attach it with clean gloves to avoid contamination.
- During filtration, if the filter clogs there is a risk that the cartridge will be expelled from the tube. It is therefore strongly recommended to hold the cartridge during filtration or to attach it to the boat or pump with adhesive tape, taking care not torisk contamination.
- It is recommended to carry out the filtration in a single operation, without stopping, as this favours filter collapse. It is also recommended to hold the tube tip and strainer under water during the entire filtration process in order not to filter air.
- If specialized equipment is used for the collection of samples (e.g. Niskin bottle or integrated sampler), it needs to be cleaned (see the above described workflow)before the sampling event to avoid contamination by external DNA.

IV. STEPWISE PROTOCOL

IV.1 Preparation before sampling

Prepare the preservation buffer

The preparation of the preservation buffer is only needed if PPs will not purchase the prepared sampling kits from SPYGEN. The preparation must be done under clean laboratory conditions to limit the possibility of DNA contamination (i.e. use gloves throughout the preparation, before starting the work wipe benches with a 10% H_2O_2 or bleach solution followed by 80% ethanol). For each sampling campaign make up a fresh batch of preservation buffer and store the buffer at room temperature.

1. Prepare all reagents and make up the right solutions if needed:

   EDTA (0.5M) [Sigma-Aldrich], Tris-HCl (1M) [Sigma-Aldrich], NaCl (0.5M), N-lauroyl sarcosine (20%) [Sigma-Aldrich], Ultrapure water

2. Combine all reagents:

For 100 mL of Preservation buffer	Final Concentration
20 mL EDTA (0.5M)	100 mM
10 mL Tris-HCl (1M)	100 mM
2 mL NaCl (0.5M)	10 mM
5 mL N-lauroyl sarcosine (20%)	1%
2 mL Ultrapure water	

3. Shake or vortex the solution to mix all reagents.
4. Adjust the pH to 7.5-8 and transfer the solution to a 100 mL volumetric flask and complete with ultrapure water to 100 mL and shake the solution to mix.
5. Using a 0.2 µm filter sterilize the buffer and divide the solution into single use aliquots and store these at room temperature.

Clean sampling equipment, collect an equipment control and prepare sampling kits

Before starting the sampling all materials, that will come into contact with the water samples before they are filtered (e.g. Niskin sampling bottles, tubing carrying the water to the filter cartridge, etc.), will need to be cleaned to remove potential contaminant DNA. **Do not** treat the materials stored in the SPYGEN sampling kits because these have already undergone cleaning procedures and additional handling is likely to increase the risk of contamination. All equipment should be washed with a detergent and thoroughly rinsed with tap water. Subsequently, contaminant DNA needs to be removed by washing equipment with a 10% H_2O_2 or a 20% commercial bleach solution. To ensure that all DNA is removed it is recommended that all equipment is exposed to the cleaning agents (H_2O_2 or bleach) for 10-20 min. Finally, rinse all equipment three times with distilled or Milli-Q water to remove any remaining cleaning agents.

Once all equipment is cleaned take 1 L of DNA free water sample (e.g. distilled or Milli-Q water) and expose the water to the cleaned surfaces of the sampling equipment. For example, transfer the sample to the cleaned sampling bottles (Niskin or otherwise), close thebottles and shake vigorously to make sure the water makes contact with all the innersurfaces. Repeat the above protocol for all cleaned sample bottles. Also expose the water to any other materials that have undergone cleaning (e.g. running the water through cleaned tubing) and afterwards collect the 1 L water sample in a clean DNA-free recipient. Proceedby filtering the 1 L sample using a 0.45 µm Sterivex filter unit. Once the sample is processed and preserved, label the filter capsule and a clean bag using the following format: “Fish”, “BEC”, “Country code”, “Date”, “Volume”. Store the sample in a dark place at room temperature. Samples can be preserved this way for up to a 3 months before DNA extractionor during the shipping process. **In no circumstances should the samples be stored below 0 °C!**Finally, let all equipment air dry (preferably not in a laboratory environment where DNA is being handled) and once dried prepare individual sampling kits containing all the required materials needed for the collection of a single sample.

IV.2 Water sampling, filtering and eDNA preservation

Key Lakes

Sampling of the shoreline

1. Get all the material ready for the collection and filtering of a single water sample (i.e. gloves, filtration cartridge and caps, absorbent paper, permanent marker or pre-printed labels, field sampling datasheet (see page 18), pen, preservation buffer, funnel, pump and tubing).
2. Put on a pair of gloves.
3. Remove the filter cartridge from its blister pack and connect the clean DNA-free tubing from the single use sampling kits to the inlet of the filter cartridge (the flow direction is indicated by an arrow on the filter cartridge). Handle the equipment carefully to ensure that the ends of the filter cartridge and tubing don’t come into contact with potentially contaminated surfaces (it is recommended to leave the end of the tubing in the clean bag) (see Figure 4).
4. Connect the second tubing (this one does not need to be clean and DNA-free) to the outlet of the filter cartridge. Insert the other end of the tubing into a recipient to collect the filtered water and measure the volume of filtered water (see Figure 4).
5. Insert the tube which is connected to the inlet of the filter cartridge into the peristaltic pump (see Figure 4).

Figure 4. Pictures showing how to: connect the clean DNA-free tubing to the filter cartridge (panel A), attach the sampling tubing to the sampling pole/stick to keep the tube below the water surface while sampling (panel B) and collect and filter water samples for the integrated sampling protocol (panel C).

6. When you have arrived at the start of the sampling transect, record the exact coordinates and time on the sampling datasheet. Next, put the end of the tube with the strainer in the water (10-20 cm under the water surface). Avoid touching the end of the tube in contact with the water with your hands. To keep the tube underwater during filtration, it may be useful to attach the tube to a sampling pole/stick covered with a plastic bag (see Figure 4). If a sampling pole/stick is used make sure to change the plastic bag between different sampling transects.
7. Start the pump and start collecting and filtering water through the cartridge. Monitor the flow during filtering in case the filtering speed needs to be adjusted.
8. After *ca*. 30 L of water has been filtered through the cartridge remove all excess water by passing air through the filtering system. Once all excess water has been removed stop the pump, disconnect the tubing from the filter cartridge and place thecartridge on a clean surface (e.g. use the clean bag which previously was used tostore the tubing).
9. Change gloves.
10. Place one of the caps over the outlet of the cartridge.
11. Place the clean DNA-free funnel in the inlet opening of the cartridge and fill the cartridge with preservation buffer (stop when the level of the preservation buffer is 0.5 cm above the filter, see Figure 2).
12. Place the remaining cap on the inlet of the cartridge and turn off the cartridge so the outlet faces upwards.

13. Remove the cap of the outlet of the filter cartridge and gently add more preservation buffer through the outlet. Stop the filling when bubbles begin to rise in the cartridge tip and replace the cap on the filter outlet.
14. Place the cartridge horizontally and shake it from left to right for 1 minute.
15. Label the cartridge and the sterile bag (see Table 2 for the standard labeling format) and place the cartridge into the sterile bag. Store the cartridge with the bag into the box that contained the cartridge and place the box in a cooling box. Make sure that all filters are stored vertically with the inlet facing upwards.
16. At the laboratory store the box containing the cartridge in a dark place at room temperature up to 3 months. **In no circumstances should the samples be stored below 0 °C!**

Sampling of the depth strata

1. Record the coordinates of the 3 stations and the time of sampling on the data sheet
2. At each location, before starting the collection of the samples rinse the sampling bottle with sampling water by moving it up and down twice from 0-20 m. This is doneto remove remaining cleaning agent (i.e. at the first sampling station) or to remove trace DNA fragments from the previous station (i.e. at the second and third sampling station). Also rinse the clean DNA-free recipient (capacity > 36 L) with some lake water from the first sampling station to remove any remaining cleaning agent.
3. Collect 2 L water with the sampling bottle from 6 water depths with an equal depth interval between them and starting at a depth of 0 m and to a few meters above the maximum depth. If the maximum depth < 70 m (e.g. 65 m) sampling depth should be adjusted accordingly (e.g. sampling at 20, 28, 36, 44, 52 and 60 m). If the maximum depth at the sampling station > 70 m samples should be collected from 20, 30, 40,50, 60 and 70 m. Combine the samples collected at all depth and all stations into one clean DNA-free recipient (capacity of *ca*. 40 L).
4. Once all samples have been collected homogenize the collected water and start filtering ca. 30 L of water through the cross flow filter cartridge following the protocol as described for the shoreline samples.

Additional Lakes

1. Record the coordinates of the first sampling site (this should be done at each site).
2. Put on gloves and take the clean DNA-free 2 L sampling bottle. Rinse the sampling bottle 3 times with lake water at the first sampling site. Also use lake water from the first sampling point to rinse the larger clean and DNA-free recipient (capacity > 30 L).
3. Collect 2 L of subsurface water sample and transfer the water to the larger recipient.
4. Move on to the next sampling points and again collect 2 L of water and combine all samples into the larger recipient. Cleaning or rinsing of the 2 L sampling bottle is not required at all sampling sites because all collected water will be combined.

   NOTE: All material must be cleaned when moving between lakes!
5. Once all 16 sampling sites have been sampled, homogenize the full 32 L of water and proceed by filtering using a cross flow filter cartridge. Detailed description on how to filter, preserve and store the samples can be found in the protocol for the collection and processing of shoreline samples for the key lakes.

Key Rivers and Additional Rivers

1. Set-up the filtering system for the collection and filter of large volumes of water using the cross flow filtering cartridge (see protocol description for the shoreline samples collected from the key lakes).
   If the direct collection and filtering of river samples is not possible, an alternative would be to collect multiple discrete water samples from the area of the main river flow and combine samples into a large clean DNA-free recipient. The protocol would thus be similar to the one described for the additional lakes (see above) with the exception that samples will be collected from a single point instead of multiple sampling sites.
2. Collect, filter and preserve samples following the earlier described protocols. For the key rivers a total of two 30 L samples will need to be collected for each sampling station while one 30 L sample per station is sufficient for the additional rivers.

V. STANDARD SAMPLING DATASHEET

For station names, coordinates and time of filtration three lines are given. For integrated shoreline samples only one name and filtration time should be provided but three coordinates should be noted indicating the start, middle and end of the transect. For the integrated depth samples three names and coordinates should be given but only one filtration time.

The sampling datasheet can also be used to record the details of three point samplescollected along one of the integrated sampling transects. However, this is only relevant for the few lakes in which both sampling protocols will be used.

Lake/River name		Sampling date
Station name(s)		
1.		
2.		
3.		
Coordinates		
	Latitude	Longitude
1.		
2.		
3.		
Time of filtration		
	Start	End
1.		
2.		
3.		
Water volume filtered		**Filter colour**
1.		
2.		
3.		
Addition data		
Conductivity		Dissolved oxygen
Temperature		pH
Wind speed		Weather

VI. APPENDIX 1: POINT SAMPLING FOR LAKE BOURGET & GARDA

Sampling design

In a selected number of lakes (i.e. Lake Bourget and Lake Garda) a point sampling approach will be used alongside the integrated approach to evaluate the benefits/limitations of both approaches. Point samples will consist of ca. 2 L water samples collected at discrete locations which will be filtered using a Sterivex filter cartridge (0.45 µm) and preserved usingthe same buffer as the integrative samples. For robust statistical comparisons between the integrated and the point sampling approach it is recommended that for each integrated sample (i.e. each shoreline transect or each integrated depth sample) three discrete point samples will be collected along the same transect or at the same locations for the shoreline and depth samples, respectively (for an example see the presentation on the Lake Garda sampling design from the meeting in Thonon-les-Bains [FisheDNA Sampling Garda JB.pptx]).

Sampling of the shoreline

For each shoreline transect collect 2 L water samples at the start, the middle and the end of the transect using a clean DNA-free sampling bottle. Ideally, samples should be collected at the same time as the integrated samples (i.e. collection of samples from a moving boat) but if this is not possible point samples could be collected afterwards by revisiting the start, middle and end of the transect. In the latter case, please ensure that appropriate geolocations are collected during the collection of the integrated sample to assure that pointsamples can be collected at the same spatial scale. Finally, samples should be stored on ice until filtering.

Sampling of the depth strata

For the selected key lakes, integrated depth samples will be collected as described previously. However, 15 L integrated depth samples will be collected at each of the three locations (e.g. collection of 2.5 L of water at 6 equal depth intervals or three 5 L integrated depth samples). For each location the 15 L water samples should be stored in a separate clean and DNA-free recipient. After sampling each location the water collected from each station will be homogenized and a 2 L subsample will be collected using a clean DNA-free sampling bottle and samples will be stored on ice until filtering using a 0.45 µm Sterivex filtercartridge (i.e. this can be done while moving between stations). The same process will be repeated at the other sampling stations and when all 2 L subsamples have been collectedthe remaining 13 L of integrated water samples can be combined into a single recipient. Homogenize the ca. 39 L of water and filter 30 L of water through the cross flow filtration capsule (i.e. VigiDNA® 0.45 µm capsule) following the earlier described protocol.

Filtration, sample preservation and storage

All collected point samples will be filtered using a Sterivex filter cartridge with a 0.45 µm pore size and sterile 100 mL syringe. For each sample use a new pair of clean gloves and remove the filter capsule and syringe from their packaging. Gently homogenize the 2 L sample and step by step filter the entire 2 L (or as much as possible) through the filter capsule. It is recommended to capture the filtered water into a measuring beaker to obtain an accurate measurement of the total volume of water filtered. After all the water has been processed remove the excess water from the filter by gently pushing air through the capsule.Once all the excess water has been removed, remove the syringe from the filter and close offthe filter outlet with a cap. While holding the inlet upwards add 2 mL of the preservation buffer to the filter capsule using a 1000 µL pipette and close the inlet of the Sterivex filter unit. Finally, double check that both caps are properly screwed onto the filter unit to avoid leaking of the preservation buffer (to absolutely make sure that no leaks will occur tightly wrap some parafilm around the ends of the filtering unit). Hold the filter horizontally and shake it from left to right for 1 minute. Label both the filter capsule and a sterile bag with thestandard labelling format given in Table 2. Place the filter capsule in the sterile bag and storethe sample in the dark in a cooling box. Samples can be stored at room temperature for weeks to 3 months. **In no circumstances should samples be stored below 0 °C!**

Additional materials check list

- Clean DNA-free sampling bottles with a capacity of 2 L (1 for each sample).
- Sterile syringe with 60 or 100 mL capacity (1 is needed for each sample).
- Sterile Sterivex filtering unit with a pore size of 0.45 µm (1 for each sample) and caps to close off the inlet and outlet (Luer-lock male and female).
- Measuring beaker with volumetric indication marks with a capacity of 2 L (used to collect and accurately measure the total volume filtered).
- Either three large recipients (capacity ≥ 30 L) with volumetric indicator marks or three sampler recipients (capacity ≥ 15 L) and one large recipient (capacity ≥ 30 L).
- Labelled sterile bags (or 50 mL falcon tubes) to store the cartridge after filtering (1 for each sample).
- 1000 µL pipette and sterile 1000 µL filter tips.
- Preservation buffer (3 mL for each sample stored in separate aliquots).
- Gloves (a minimum of 2 pairs per sample).
- Field sampling datasheet and a pen.
- Parafilm (optional but recommended).
- Scissors (optional).

Stepwise protocol

Sampling of the shoreline

1. Follow the general protocol for the collection of integrated shoreline water samples but simultaneously a second person will be collecting smaller volumes (2 L) of water for eDNA analyses.
2. Before starting the sampling label three sampling bottles with the name of the sampling station (identical to the name used for the transect) followed by the sampling location relative to the transect (i.e. start, middle or end).
3. While sampling equipment is being prepared for the integrated sample the second person puts on a pair of gloves, rinses the sampling bottle with lake water 3 times and collects a 2 L water sample from the starting point of the sampling transect. Water samples could be collected from the first 5-10 cm of the water surface simply by keeping the sampling bottle horizontally and submerging it to the desired depth.
4. Close the sampling bottles properly and store the collected samples in the cooling box with ice (or ice packs) to avoid further degradation of the eDNA.
5. Repeat the process at the middle and end of the sampling transect. Make sure you change gloves in between samples and that you start rinsing the respective sampling bottles before *ca*. 15 and 30 L of water has been collected for the integrated sample. Once *ca*. 15 and 30 L has been collected proceed with collecting the 2 L point sample (preferably without stopping the boat, it can however be slowed down to make sure the collection happens safely). Make sure to record the coordinates for all the collected point samples.

   An alternative process would be to first collect the integrated sample while keeping an accurate track of the transect (e.g. through a mobile app like OruxMaps). After the collection of the integrated sample; revisit the start, middle and end of the transect; rinse the sampling bottles with lake water; and collect and store the 2 L watersample.
6. Once all point samples have been collected from a single transect you may proceed with filtering the samples if adequate time and space is available on the boat to work in a clean manner. Alternatively, samples (stored on ice) could be transferred to the lab at the end of the day and filtering can be performed in the laboratory.
7. Put on a pair of new gloves and remove the syringe and Sterivex filter unit from their blister packs (handle with care to avoid either ends of the syringe or Sterivex to touch contaminated surfaces).
8. Gently homogenize the 2 L water sample, open the sampling bottle and fill thesyringe with water (verify that the neck of the bottle is larger than the syringe's diameter).

9. Connect the syringe and the Sterivex filter and slowly filter through the water sample. Make sure to collect the filtered water so afterwards an accurate estimate ofthe total volume of filtered water can be obtained.
10. Repeat the process until the complete 2 L water sample has been processed (preferred) or the filter is clogged and no more water can be passed through.
11. Once the filtering has been completed remove the excess water from the Sterivex unit by passing air through the filter.
12. Screw the cap on the filter outlet (make sure the outlet is properly sealed).
13. Using a 1000 µL pipette and the single use aliquot of preservation buffer add 2 mL of preservation buffer to the Sterivex filter. Make sure to insert the pipette deep enough into the Sterivex filter!
14. Screw the cap on the filter inlet (make sure the inlet is properly sealed). Shake the Sterivex filter horizontally for approximately 1 minute.
15. As an extra precaution to avoid leaking of the preservation buffer it is recommended to apply some parafilm at the ends of the Sterivex filters.
16. Label the filter unit and the clean plastic bag (see Table 2 for standard labelling formats) and place the filter unit in the plastic bag. Store filters in the dark at room temperature up to 3 months. **In no circumstances should samples be stored below 0 °C!**

Sampling of the depth strata

1. Follow the general protocol for taking an integrated depth sample from the key lakes but instead of collecting a 12 L depth integrated sample at each station now collect a 15 L depth integrated sample at each station. Also store depth integrated samples collected at each station in a separate clean DNA-free recipient.
2. Once a depth integrated sample has been collected from one of the stations homogenize the collected water and use a clean DNA-free 2 L sampling bottle to take a 2 L subsample.
3. Store the 2 L subsamples in a cooling box (with ice or icepacks) until further processing.
4. Once all three stations have been sampled and 2 L subsamples have been collected and stored, combine the remaining water (*ca*. 13 L for each station) into one single clean DNA-free recipient. Homogenize the large water volume and proceed by filtering 30 L of the water using the previously described protocol.
5. Filtering, preserving and storing of the 2 L subsamples will follow the general protocol as described for the shoreline samples.

VII. APPENDIX 2: POINT SAMPLING FOR LAKE MONDSEE & STARNBERG

Sampling design

At Lake Mondsee and Starnberg a high effort point sampling strategy will be used to provide a direct comparison between eDNA and traditional fish biodiversity assessment methods for lakes. Traditional methods consist of electrofishing in the littoral zone combined with the deployment of benthic and pelagic gillnets. Water samples (5 L) for eDNA based analyses will be collected at each electrofishing stretch and gillnet location. After collection, the samples will be brought to the laboratory and filtered through glass fibre filters (⌀ 47mm, GF/C) and stored at -20° until DNA extraction.

Sampling of the shoreline (electrofishing stretches)

Water samples should be collected in the middle of the traditional electrofishing stretches. Thus, the number of sampling sites has to be chosen corresponding to the lake surface area. Lakes < 4km² surface area need at least 4 lakeshore sampling sites (electrofishing stretches), lakes > 4km² surface area need 1 site for each km². The sampling sites should represent all the different lakeshore habitats. A 5 litre water sample (using a Schindler-Patalas sampler or a DNA free recipient) will be collected at each sampling site 10-20 cm below the surface. The samples should be stored in a clean, DNA free recipient. The coordinates of each sampling location have to be noted and cooling of the samples until filtration needs to be ensured.

Sampling of benthic gillnet locations

Sampling in the benthic zone is used to detect fish species inhabiting the littoral and the benthic zone. The number of gillnet locations used for the traditional sampling depends on the maximum depth and the surface area of the lake according to the standardized European guideline for gillnet fishing (CEN: EN 14757:2015 Water quality — Sampling of fish with multi-mesh gillnets). A single 5 litre point sample (Schindler-Patalas sampler) should be taken at each benthic gillnet location approximately at the same depth as the net would be placed (1-2 m above the bottom to avoid disturbance of the substrate). The samples should be stored in a clean, DNA free recipient. The coordinates of each sampling location have to be noted and cooling of the samples until filtration needs to be ensured.

Sampling of pelagic gillnet locations

Sampling in the pelagic zone is used to detect fish species inhabiting the pelagic zone. For lakes with a surface area of ≤ 5 km² one sampling site (deepest point) should be sampled, forlakes between 5 - 10 km² the deepest point and an additional random site should besampled. For lakes > 10km² the deepest point as well as two additional random sites should be sampled (EN 14757:2015). Integrative sampling of the whole water column (0-70m) couldbe done by:

1. Taking e.g. three point samples from different depths of the epilimnion/metalimnion (at 0, 10 and 20m) put them together in a DNA free recipient and take a sub-sample (5 L) from that recipient. In addition, do the same for the hypolimnion e.g. take 4 point samples (at 20, 35, 50 and 65m) put them into a DNA free recipient and take a sub-sample from that recipient (5L).
2. Taking two 5 L depth integrated samples (0-20 m and 20-70 m) at each sampling location using an integrative sampler (e.g. Hydrobios IWS 3, Niskin bottles). The samples should be stored in a clean, DNA free recipient.

The coordinates of each sampling location have to be noted and cooling of the samples until filtration needs to be ensured.

Additional materials check list and precautions during sampling

Additional materials check list

- Schindler-Patalas sampler and/or Integrated sampler (IWS Hydrobios or Niskin bottles)
- Winch
- Multi-probe
- Gloves
- Glass-fibre discs (GF/C ~ 1.2 µm) for filtration (1 per sample)
- Vertical filtration device
- DNA free recipients ≥ 5L for each point sample and ≥ 20L for mixing of point samples from different depth strata.
- Marker pen+ label OR pre-printed label for sample identification
- Field protocol + pen
- Qiagen Power Water Kit for eDNA extraction
- GPS-device
- Cooling box with ice / ice packs for sample transport

Precautions during sampling

- Store all materials inside clean bags or containers to reduce contamination risk.
- Wear gloves during the whole sampling and filtration procedure.
- Change gloves between each sampling location.
- Prior to sampling, recipients need to be washed (H_2O_2 10%) and rinsed with distilled water.
- During the handling try to reduce exposure to sunlight if possible.
- Avoid sampling during/shortly after heavy rain/flood events due to increased particle transport which may cause clogging of the filter.
- Sampling devices (samplers) need to be washed with H_2O_2 (10%) between each sampling location and rinsed with lake water at the new station several times.
- Glass fiber filters need to be autoclaved prior to use.
- Vertical (open) filtration device needs to be cleaned (H_2O_2 10%) and rinsed with MQ after each filtration.
- Working surface needs to be cleaned with H_2O_2 (10%) to avoid cross contamination.

Stepwise protocol

1. Take all the (sub-) samples (5L) to the laboratory.
2. Keep them cooled during transport, avoid exposure to sunlight.
3. Make sure the working surface is cleaned before starting the filtration.
4. Wear gloves during the whole procedure.
5. Place autoclaved glass-fiber discs (1.2 µm) on vertical filtration device (needs to be sterilized with 10 % bleach or 10% H_2O_2 and afterwards rinsed with MQ water prior to use).
6. Fill sample into filtration device.
7. Filter the water sample through the glass-fiber filter.
8. If possible, 5 liter need to be filtered through each glass-fiber filter. If the filter clogs before reaching the 5L mark, note down the final volume filtered.
9. Do not let the filter dry out completely to avoid degradation of DNA.
10. Fold the filter with pincers and put it into an Eppendorf tube.
11. Label the tube.
12. Store the tube (containing the filter) at -20°C.

13. If necessary, complete the lab/field sheet.
14. Clean the filtration device with 10 % bleach or 10% H_2O_2 and rinse it with with mQ water before you filter the next sample.