Interreg
Alpine Space
Eco-AlpsWater
European Regional Development Fund

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Technical guidelines for eDNA monitoring in Alpine waters

2021

FOR STAKEHOLDERS AND END-USERS

Eco-AlpsWater

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

Protocols for all eDNA monitoring steps! The present protocols are state-of-the-art today, but due to continuous revisions and technical improvements, they will be optimized in the near future.

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PUBLISHED by National Institute of Biology

Electronic edition
Ljubljana, 2021

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Kataložni zapis o publikaciji (CIP) pripravili v Narodni in univerzitetni knjižnici v Ljubljani
COBISS.SI-ID 89004291
ISBN 978-961-7144-09-3 (PDF)

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Contents

I. State of the art of methods for the analysis of environmental DNA in lakes and rivers

Freshwaters in lakes and rivers provide goods and services of critical importance to human societies everywhere; the protection and preservation of these aquatic ecosystems is therefore a major challenge. Aquatic biomonitoring now underpins much of the management and conservation of freshwaters and has become an essential task in Europe as a consequence of strong anthropogenic pressures affecting the health of lakes and rivers. An effective evaluation of the quality/status of aquatic ecosystems requires comprehensive data on various freshwater organisms (from micro-algae to fish) used as indicators of the ecosystem health. The required biodiversity metrics are obtained by collecting bioindicator organisms, which are identified at the species level, to constitute taxonomic lists and subsequent quality indices. These approaches require high level of taxonomic expertise and are generally invasive (e.g. electrofishing), time consuming, technically complex and thus expensive to deploy on large temporal and spatial scales. High-throughput genetic screening methods such as environmental DNA (eDNA) metabarcoding have been recently proposed as a solution to these shortcomings. Such new generation biomonitoring has many advantages over the traditional approach in terms of speed, comparability and costs, offering the possibility to monitor aquatic biodiversity with a non-invasive and rather easy-to-standardize approach. eDNA is the DNA collected from environmental samples (here water, or biofilms), including both DNA found in living cells (e.g. bacteria, micro-algae) and DNA released in the environment by all types of organisms (e.g. fish). From eDNA samples, short DNA regions called “barcodes” can be amplified, sequenced using High-Throughput Sequencing (HTS) technologies and compared to a reference library allowing to identify taxa initially present in the sampled water/biofilms. Though eDNA metabarcoding has been recognized as highly promising for next generation biomonitoring, the associated methodologies are not standardized so far and each step of the eDNA workflow needs to be normalized and validated at the European level before it can be implemented for routine lake and river monitoring. One of the main aims of the Eco-AlpsWater project is to formalize standard eDNA protocols for both bacteria, micro-algae and fish, and to implement those protocols at the alpine scale for pilot lakes and rivers. As highlighted by the Fig. 1, the state of the art protocols we have referred here include information for each step of the eDNA workflow, i.e. sampling, DNA extraction and laboratory preparation (including selections of barcodes and PCR amplification), as well as sequencing and bioinformatic treatments allowing to produce taxonomic lists. Though including a common number of steps, the protocols have to be adapted to the different type of biological elements. An illustrative example, with the major steps and methods applied for fish, is given in Fig. 2. From the synthesis provided in this technical document the Eco-AlpsWater consortium has made some methodological choices that help to formalize appropriate eDNA strategies transferable for freshwater routine monitoring. The main questions addressed in this bibliographic synthesis are: (i) when, how and where to sample for eDNA, (ii) how to concentrate and preserve eDNA, (iii) what is the most appropriate DNA extraction method to apply, (iv) what are the barcodes to use according to the objective of biomonitoring, and (v) what are the sequencing

technologies and bioinformatics pipeline to select for obtaining robust taxonomic inventories for the studied biological groups.

All the protocols described are among those proposed by the Eco-AlpsWater consortium to promote the implementation of eDNA analysis with HTS methods in biomonitoring and ecological assessment of lakes and rivers.

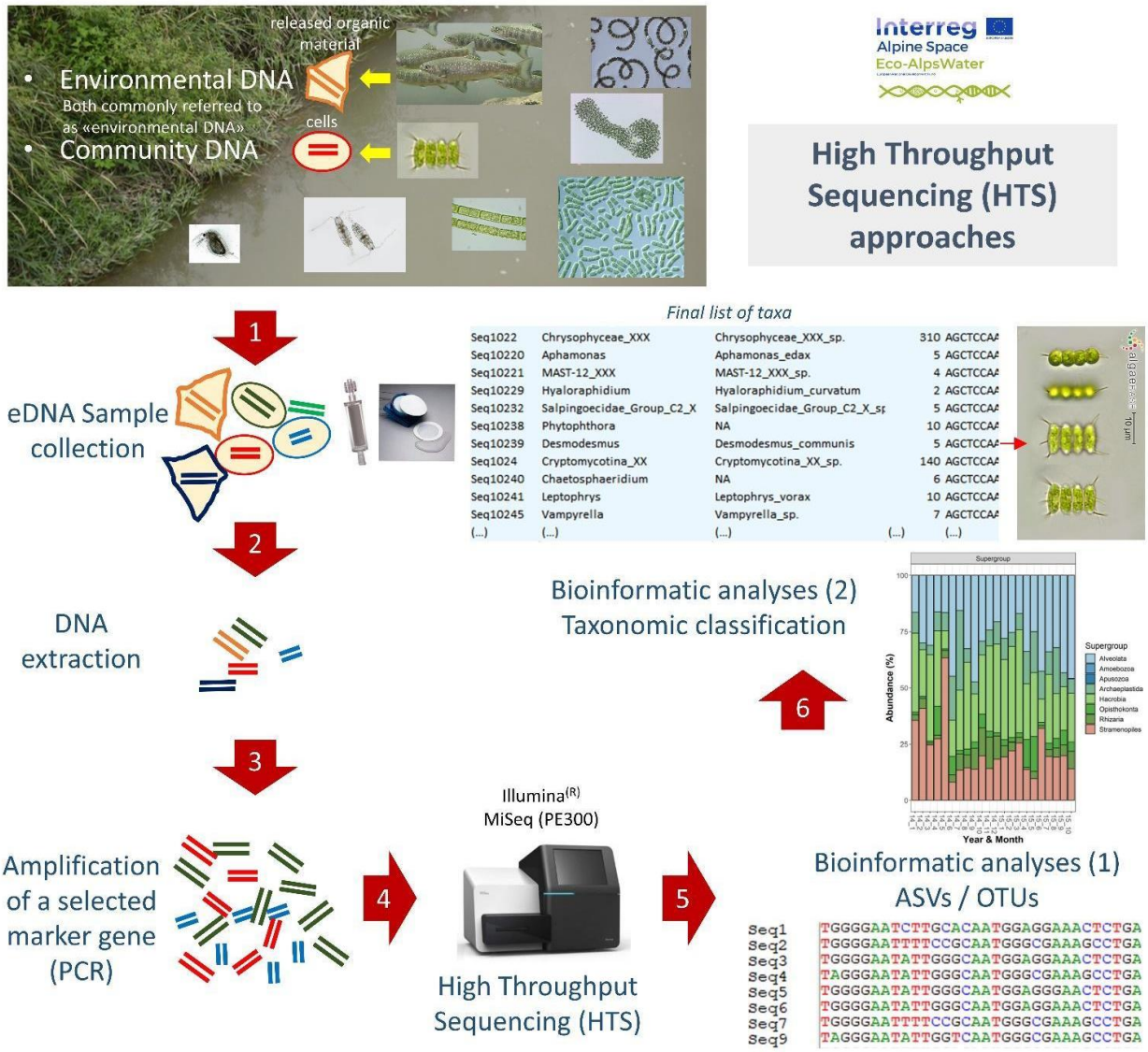

Fig. 1. Synthetic representation of the major common steps applied in the analysis of eDNA in freshwater ecosystems (lakes and rivers). The figure includes an example of amplification, sequencing and classification of protist organisms (red, blue and dark green DNA sequences), but the steps are the same for other aquatic organisms. However, each biological element requires specific adaptations of the procedures in all six steps of HTS (see e.g. Fig. 2).

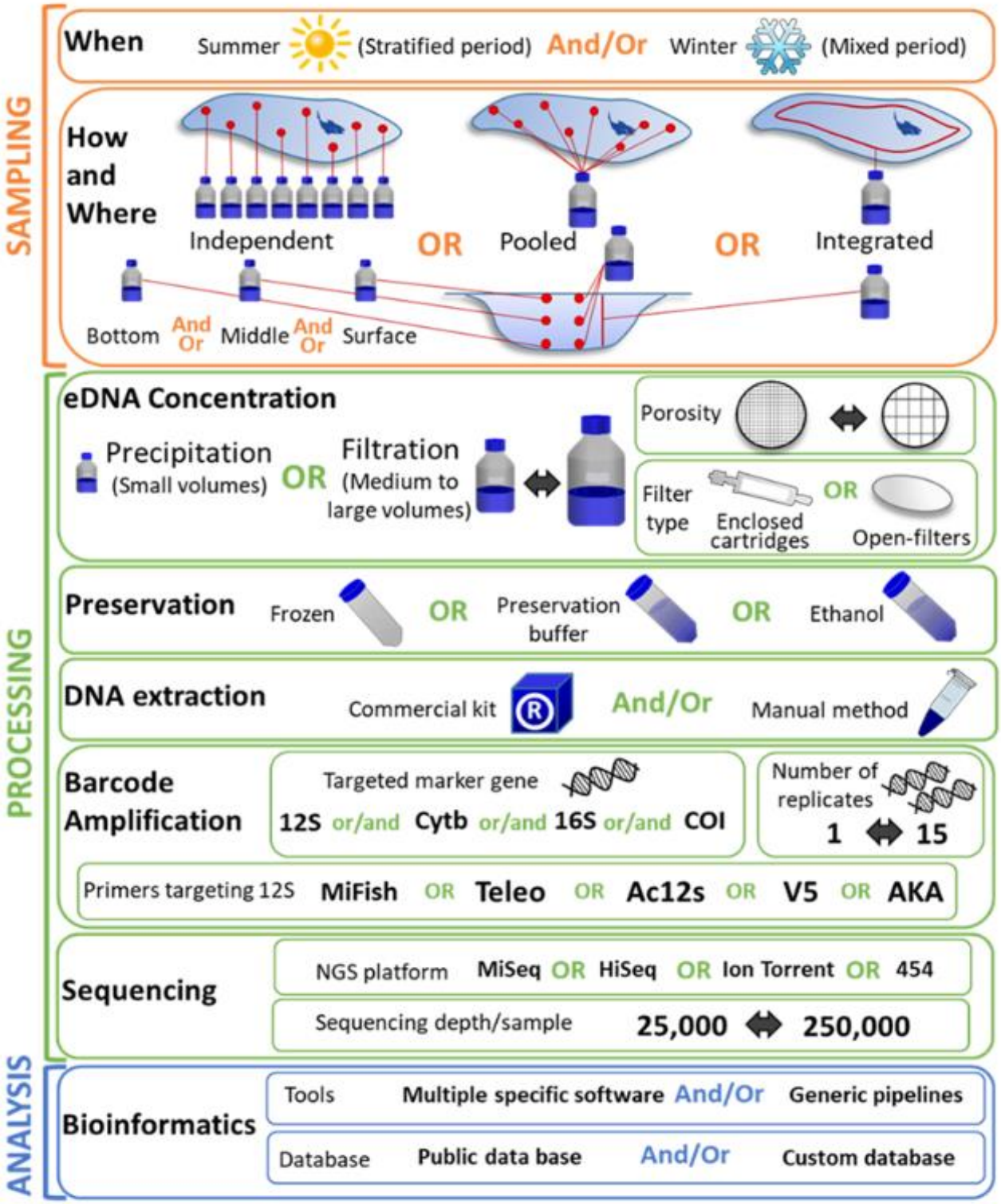

Fig. 2. Synthetic representation of the major steps and methods applied in the literature for fish eDNA metabarcoding in freshwater ecosystems (lakes and rivers).

II. Protocols for sampling (Fig. 1, step 1)

Plankton sampling

Lake plankton sample collection from the field for downstream molecular analysis

The objective of this protocol is to provide a reliable and replicable method for the sampling of lake micro-plankton to be used for downstream DNA analysis. The application proposed here, in the context of Eco-AlpsWater, aims at comparing DNA inventories to traditional phytoplankton inventories and at characterizing more broadly the micro-planktonic diversity through eDNA analysis (including bacteria). The sampling strategy is similar to that used for classical phytoplankton survey focusing on the euphotic zone (Fig. 3), however the procedure for filtration and preservation is adapted for DNA samples.

Protocol:
dx.doi.org/10.17504/protocols.io.xn6fmhe

Short movie presenting the plankton sampling for eDNA analyses
https://www.youtube.com/watch?v=du5dfjNQr1E

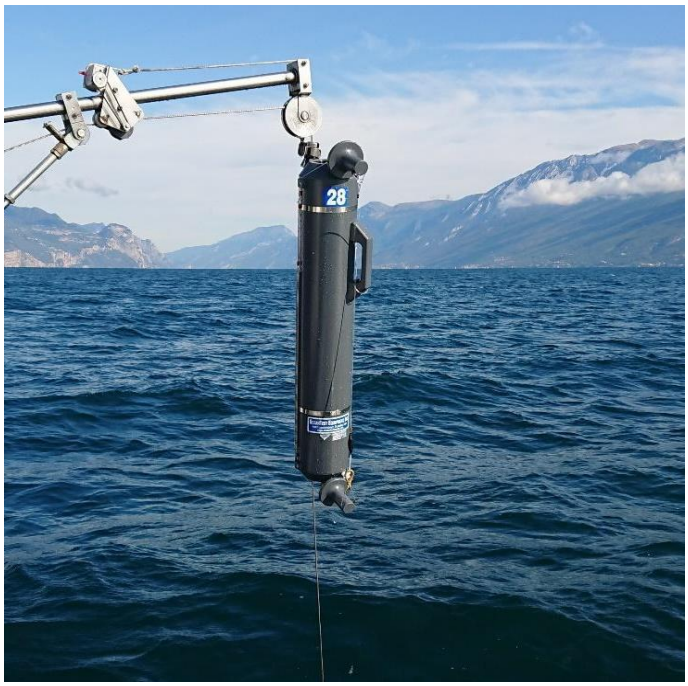

Fig. 3. Niskin bottle used for water sampling at precise depths in Lake Garda.

Biofilms sampling in rivers & lakes

River biofilms sampling for both downstream DNA analysis and microscopic counts

The objective of this protocol is to provide a reliable and replicable method for the sampling of river 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 (FprCEN/TR 17245) for the analysis of benthic diatoms from rivers (e.g. Fig. 4) and lakes. The application proposed here in the context of Eco-AlpsWater aims at comparing DNA inventories to traditional inventories (microscopy).

Protocol for lake biofilm sampling:
dx.doi.org/10.17504/protocols.io.br2xm8fn

Protocol for river biofilm sampling:
dx.doi.org/10.17504/protocols.io.ben6jdhe

Short movie presenting the biofilm sampling for eDNA analyses
https://www.youtube.com/watch?v=_6Q48nSMjNA

Fig. 4. Bistrica river, biofilm sampling with sterilized brush and gloves.

Fish eDNA sampling in rivers & lakes

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

The objective of this protocol is to provide a reliable and replicable method for the sampling of lake and river fish (e.g. Fig. 5) to be used for downstream DNA analysis. The application proposed here, in the context of Eco-AlpsWater, aims at comparing DNA inventories to traditional fish inventories. Three different sampling design approaches, varying between filter types used, sample number and filtered volume per sample are briefly described (cf. Fig. 2). Each of these sampling methods and associated DNA extraction procedures (described in section III) have their advantages and disadvantages, and it depends on the research question at hand which approach is most suitable. However, these methods and protocols are to be considered as still under development. All members of the Eco-AlpsWater consortium have contributed to the protocol optimization.

Protocol:
https://www.alpine-space.org/projects/eco-alpswater/final-results-and-deliverables/d.t1.1.2---4-lake-river-fish-edna-sampling.pdf

Fig. 5. Fish Perca fluviatilis.

III. Protocols for eDNA extraction (Fig. 1, step 2)

Plankton DNA extraction

Eco-AlpsWater protocol applied for Sterivex filters using the DNeasy® PowerWater Sterivex QIAGEN Kit

This protocol is part of the DNA workflow applied in the Eco-AlpsWater Project to characterize the diversity of plankton in lakes. Conventionally, phytoplankton analysis has been done using microscopic observations, but High Throughput Sequencing (HTS) technologies provide potential for rapid examination of environmental samples with the capacity to consider a large diversity of taxa that were traditionally not taken into account in traditional planktonic surveys. The methodological step described here is the extraction of DNA. This is a critical step for obtaining relevant results; because DNA is stored within cells (see figure below) and methods for cell lysis and DNA isolation need to be efficient to allow unbiased nucleic acid retrieval even from species with tough cell walls. For the Eco-AlpsWater project, plankton sampled in lakes is filtered on Sterivex® cartridges (Sterivex® GP 0.22 µm) and stored at -20°C, as described in the protocol below. The methodology chosen for DNA extraction is therefore adapted to the type of material/filter used for plankton collection (i.e. Sterivex® cartridge). The protocol presented below uses the DNeasy® PowerWater Sterivex® Kit (QIAGEN) with specific modifications adapted to plankton DNA extraction.

Protocol:
dx.doi.org/10.17504/protocols.io.bvgzn3x6

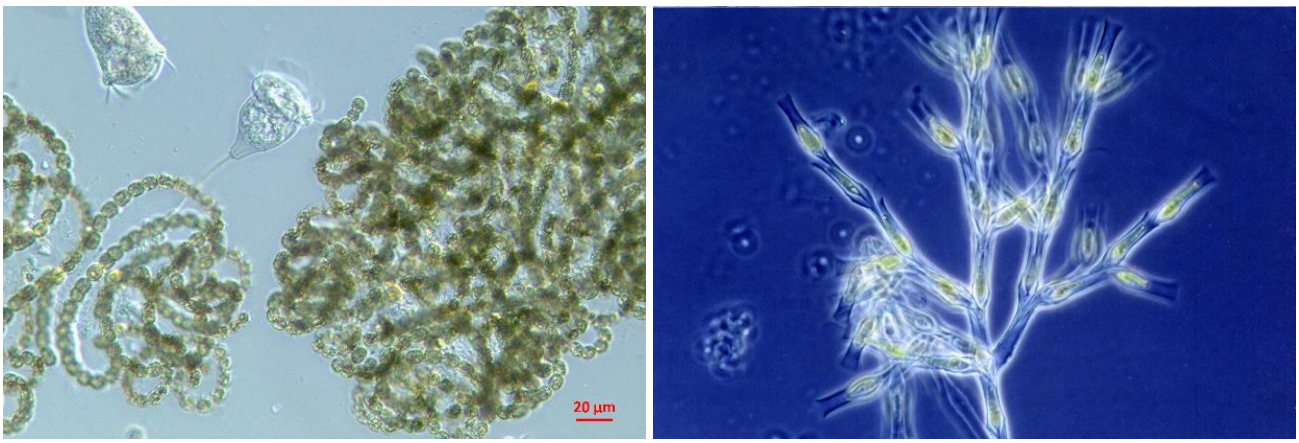

Fig. 6. Phytoplankton from Lake Garda. Left, filaments of the cyanobacterium Dolichospermum lemmermannii with attached vorticellids. Rigth, the chrysophyte Dinobryon divergens. DNA extraction provides genomic DNA from all the organisms present in one sample.

Biofilms DNA extraction

Eco-AlpsWater protocol applied for biofilms using

the NucleoSpin® Soil kit (MACHEREY-NAGEL)

The methodological step described here is the extraction of DNA, a critical step for obtaining relevant results since molecular inventories might be influenced by the DNA extraction method used. The methodology for DNA extraction of biofilms was identified by testing 5 different methods based on various types of cell lysis and DNA purification from pure diatom cultures and samples from lakes and rivers. For the Eco-AlpsWater project, after being sampled in lakes or rivers (e.g. Fig. 7) biofilms are stored in 50 mL falcon tubes in ethanol at 4°C, and for a maximum of 3 months before DNA extraction (the extraction should preferably be done in the month following the sampling). The DNA extraction protocol presented below has been used in several recent studies focused on the application of diatoms metabarcoding; this extraction is based on a protocol adapted from the NucleoSpin® Soil kit (MACHEREY-NAGEL) with specific modifications for biofilm DNA extraction.

Protocol:
dx.doi.org/10.17504/protocols.io.bd52i88e

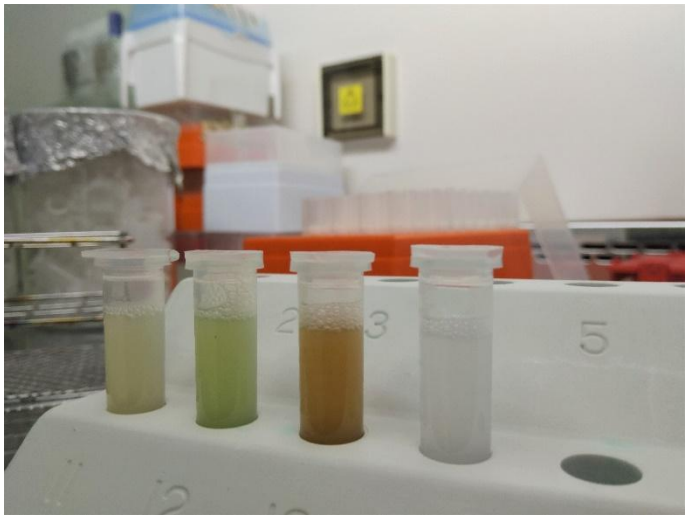

Fig. 7. DNA extraction from biofilm of different water environments.

Fish eDNA extraction (3 methods)

(1) Eco-AlpsWater protocol applied for fish DNA extraction from VigiDNA® filtration cartridge using an adaptation of the NucleoSpin® Soil kit (MACHEREY-NAGEL)

The choice of the methodology for fish DNA extraction is based on previous studies, with some adaptations for the Eco-AlpsWater project. For the integrated fish eDNA sampling approach within the Eco-AlpsWater project, water samples are collected with a VigiDNA® (0.45-µm) filtration cartridge, adapted to treat large volumes of water (30 liter). After filtration, a preservation buffer is added to the cartridge that is stored at room temperature until extraction, which should be done within 1 month after sampling. The DNA extraction protocol used for this approach was adapted from the NucleoSpin® Soil kit (MACHEREY-NAGEL) with specific modifications. The testing of the protocol was done in the context of the Eco-AlpsWater project (Eco-AlpsWater protocol applied for fish DNA extraction from VigiDNA® filtration cartridge using an adaptation of the NucleoSpin® Soil kit (MACHEREY-NAGEL).

Protocol:
https://www.alpine-space.org/projects/eco-alpswater/deliverables-final/dt1.1.2.--8.2-fish_dna_extraction_vigidna.pdf

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

The choice of the methodology for fish DNA extraction is based on previous studies, and adapted to Sterivex® filter cartridges. For the fish eDNA point-sampling (Sterivex®) approach within the Eco-AlpsWater project, water samples (2 liters) are collected with Sterivex® filtration cartridges (0.45-µm), after the filtration, the cartridges are filled with preservation buffer and stored at room temperature until DNA extraction. The extraction should be done within 1 month after the sampling. The DNA extraction protocol presented below is based on a protocol adapted from the NucleoSpin® Soil kit (MACHEREY-NAGEL) with specific modifications (Eco- AlpsWater protocol applied for fish DNA extraction from Sterivex cartridge preserved with preservation buffer and using the NucleoSpin® Soil kit).

Protocol:
https://www.alpine-space.org/projects/eco-alpswater/deliverables-final/dt1.1.2.--8.1-fish_dna_extraction_sterivex.pdf

(3) Fish DNA extraction from glass fiber filter discs using the DNeasy PowerWater® Kit

Due to comparison reasons, an additional methodology for fish eDNA extraction is used that has been proven effective in previous tests, carried out within the Eco-AlpsWater project. For the fish eDNA point sampling (glass fiber filters – GFC) approach, water samples (5 liters) were collected in alpine water bodies (e.g. Figure 8) and filtered through GFC filters (1.2 µm). After filtration, samples are stored at -20 °C until DNA extraction. DNA extraction is performed according to the protocol described by the manufacturer (Qiagen) for the analysis of filter membranes (including GFC) using the DNeasy PowerWater kit.

Fig. 8. Juvenile brown trouts (Salmo trutta) in an Alpine river.

IV. Protocols for library preparation (Fig. 1, step 3-4)

Library protocols for diatoms

PCR amplification of rbcL gene for downstream bioinformatic analyses and taxonomic classification of diatoms (Bacillariophyta, Fig. 9)

Different studies have already revealed the potential of diatom metabarcoding applications for freshwater quality assessment. The choice of the marker gene and barcode region is key for obtaining relevant inventories of diversity and precise taxonomic assignment. For benthic diatoms, the rbcL gene has proved to be an appropriate taxonomic marker for biomonitoring and a well-curated barcode reference library is already available to assign species names to rbcL sequences (R-Syst::diatom). In the Eco-AlpsWater project, biofilm samples in rivers and lakeshores are collected and DNA is extracted as described in the previous sections. Below, we present the different steps of the DNA based workflow (i.e. PCR amplification of selected barcodes, and wet lab methods to prepare DNA library for downstream MiSeq Sequencing). This protocol has been used in recent studies where diatoms metabarcoding has been used for ecological assessment of rivers.

Protocol:
dx.doi.org/10.17504/protocols.io.bd94i98w

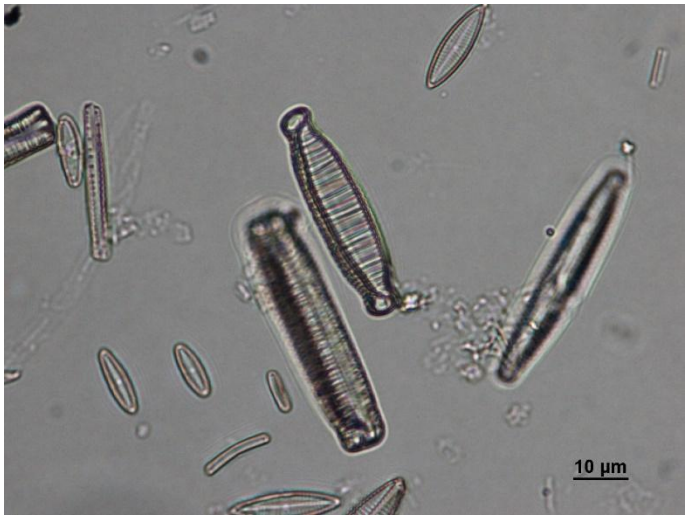

Fig. 9. Diatom species under light microscope (Diatoma, Achnanthidium, Navicula).

Library protocols for bacteria

PCR amplification of 16S rRNA genes for downstream bioinformatic analyses and taxonomic classification of bacteria and cyanobacteria

This protocol provides the basic elements that have been used for the identification of bacteria within the project Eco-AlpsWater. Analyses have been applied to DNA extracted from samples collected in the water column of lakes and biofilms collected in rivers and lakeshores (e.g. Fig. 10). The marker used for the identification of bacteria is the 16S rRNA gene, which is still widely used in the taxonomic determination and phylogenetic analyses of bacterial communities. In particular, PCR amplification of this marker in the genomic DNA extracted from environmental samples has been performed by targeting a ~ 460 bp (base pairs) fragment in the variable regions V3–V4. Besides the whole range of bacterial classes, the libraries prepared this way includes also DNA reads from cyanobacteria, which are one of the most crucial biological elements included in the environmental biomonitoring and in the biomonitoring of waters intended for drinking and recreational use. The bacterial primer set includes: 341F (5' CCTACGGGNGGCWGCAG 3') and 805Rmod (5' GACTACNVGGGTWTCTAATCC 3') with overhang Illumina adapters. This pair of primers has been widely used in the assessment of bacterial biodiversity in aquatic environments worldwide. The preparation of library followed a standard procedure described in the protocol indicated below.

Protocol:
https://www.alpine-space.org/projects/eco-alpswater/deliverables-final/d.t1.3.1-10-validated_library_prep_16s.pdf

Fig. 10. Cyanobacterial bloom of Planktothrix rubescens in Lake Ledro (Italian Alps).

Library protocols for protists

PCR amplification of 18S rRNA genes for downstream bioinformatic analyses and taxonomic classification of protists (including microalgae)

Protists are a polyphyletic assemblage of eukaryotic organisms that includes groups that are more closely related to plants, fungi or animals than they are to other protists (e.g. Fig. 11). Besides heterotrophic protists and microscopic fungi, photosynthetic and mixotrophic protists, or "algae", are scattered within many supergroups along with many other protozoans, with the exception of Archaeplastida, which form a group of their own. This protocol provides the basic elements that have been used for the identification of protists within the project Eco-AlpsWater. Analyses were applied to DNA extracted from samples collected in the water column of lakes and biofilms collected in rivers and lakeshores (see previous sections). The marker used for the identification of protists is the 18S rRNA gene. PCR amplification of the 18S rRNA genes is performed by targeting a ~380 bp fragment of the 18S rRNA gene variable region V4 using the specific primer set: TAReuk454FWD1 (5' CCAGCASCYGCGGTAATTCC 3') and TAReukREV3_modified (5' ACTTTCGTTCTTGATYRATGA 3'). This pair of primers has been widely used in the assessment of microeukaryotic biodiversity in aquatic environments. The preparation of the 18S rRNA gene library followed a standard procedure described in the protol indicated below.

Protocol:
https://www.alpine-space.org/projects/eco-alpswater/deliverables-final/d.t1.3.1-11-validated_library_prep_18s.pdf

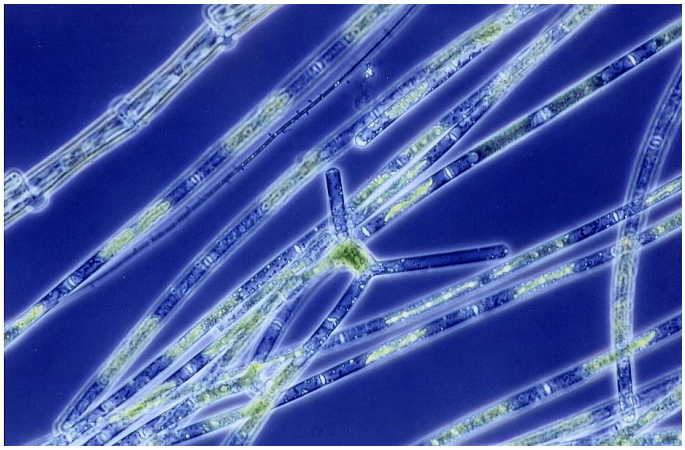

Fig. 11. The green microalga Mougeotia *sp. from Lago di Garda, Italy; filaments are about 7 µm wide.*

Library protocols for fish

PCR amplification of 12S rRNA genes for downstream bioinformatic analyses and taxonomic classification of fish

The aim of this document is to provide a detailed description of the Illumina library preparation protocol for eDNA metabarcoding analyses of freshwater fish communities (e.g. Fig. 12), previously assessed and verified through an intercalibration test. This protocol has been used at the Sequencing and Genotyping Platform at FEM for the analysis of the samples collected in 2019 within the framework of Eco-AlpsWater project. The protocol in its present form, however, is still open to improvements and the evaluation of the replicability and robustness of approaches based on different protocols is still under testing.

Protocol:
https://www.alpine-space.org/projects/eco-alpswater/deliverables-final/d.t1.1.2.-12library_preparation_12s.pdf

Fig. 12. European eel (Anguilla anguilla), a snake-like, migratory fish species.

V. Protocols for bioinformatic analyses of DNA reads (Fig. 1, step 5-6)

Bioinformatic pipelines for diatoms (RbcL gene)

Diatom DNA metabarcoding bioinformatics pipeline "Mothur" software, Miseq, rbcL 312 bp

This protocol describes in details the main steps of the bioinformatic processes applied to analyse high throughput sequencing (HTS) data, in particular for diatoms metabarcoding. Diatoms are one of the most relevant biological elements in lakes and rivers (e.g., Fig. 13).

Protocol:
https://www.alpine-space.org/projects/eco-alpswater/deliverables-final/dt1.1.3.--1-bioinformatic-diatoms.pdf

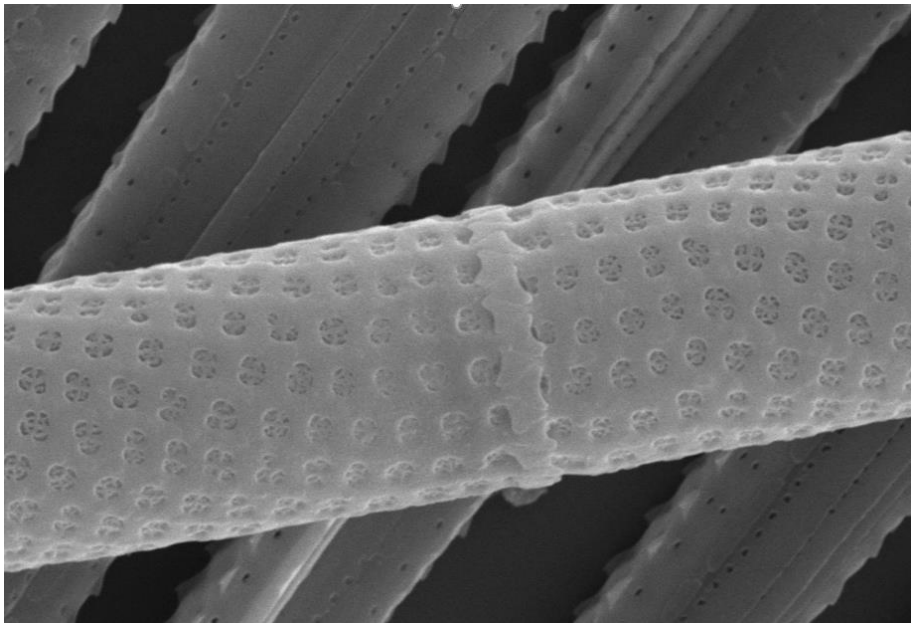

Fig. 13. Scanning electron micrograph of Aulacoseira granulata and (background) *Fragilaria crotonensis* from Lake Garda.

Bioinformatic pipeline for bacteria (16S rRNA gene)

Bioinformatic analysis of bacteria (including cyanobacteria) using the 16S rRNA gene and a DADA2 pipeline

This protocol details the main steps of the bioinformatics pipeline applied to analyse the high throughput sequencing (HTS) 16S rRNA gene data, specifically for bacteria and cyanobacteria determinations (Fig. 14). The pipeline is based on the identification of exact sequences (Amplicon Sequence Variants, ASVs) using the DADA2 approach. The protocol and test files can be downloaded from Zenodo.

Protocol:
https://doi.org/10.5281/zenodo.5232772

Test files:
https://doi.org/10.5281/zenodo.5215815

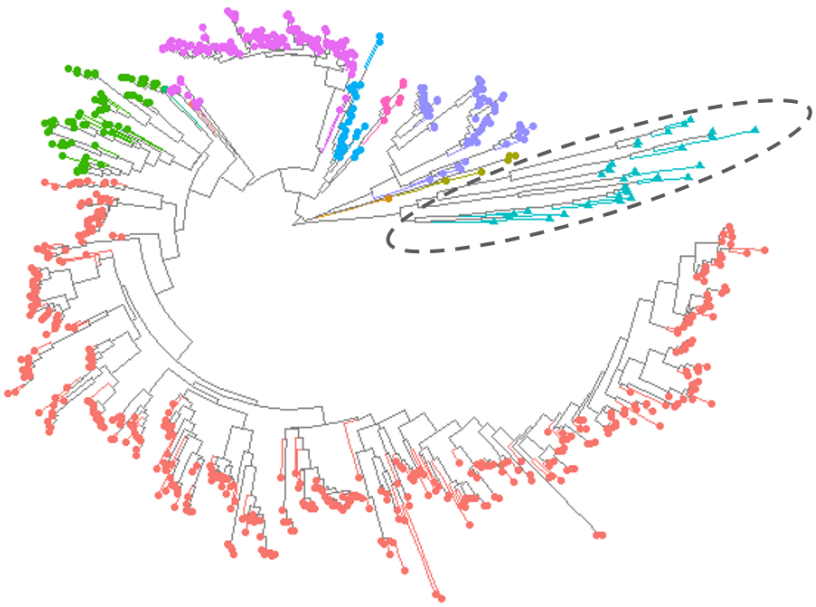

Fig. 14. Phylogenetic tree based on the alignment of the 16S rRNA gene (approx. 400 bp) obtained with HTS techniques in the Alpine Space region. Only the most abundant sequences (ASVs) were included in the analysis. Compared to traditional techniques, environmental metagenomics approaches allow to explore previously undetectable groups, such as the non-photosynthetic bacteria (grey dashed line). Other more numerous groups include Cyanobacteriales (orange), Limnotrichales (green) and Synechococcales (purple).

Bioinformatic pipelines for protists (18S rRNA gene)

Bioinformatic analysis of protists (including microalgae) using the 18S rRNA gene and a DADA2 pipeline

This protocol details the main steps of the bioinformatics pipeline applied to analyse the high throughput sequencing (HTS) 18S rRNA gene data, specifically for protists and microalgae metabarcoding (Fig. 15). The pipeline is based on the identification of exact sequences (Amplicon Sequence Variants, ASVs) using the DADA2 approach. The protocol and test files can be downloaded from Zenodo.

Protocol:
https://doi.org/10.5281/zenodo.5233527

Test files:
https://doi.org/10.5281/zenodo.5215919

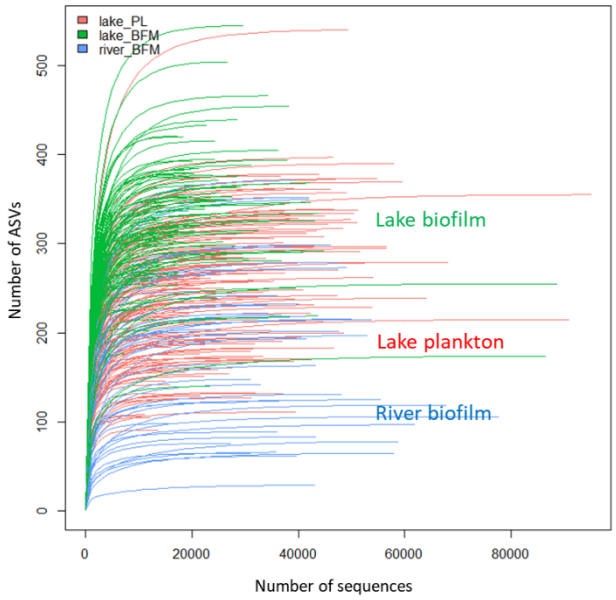

Fig. 15. Rarefaction curves of protists and fungi showing the increase in the number of ASVs with the increase in the number of sequences. Rarefaction is used to estimate the ASVs richness for a given number of sequences, allowing normalization of abundances across samples. In the figure, each curve corresponds to a single sample. On average, the largest numbers of ASVs are found in the biofilm of lakes.

VI. Harmonizing approaches in the European Community and Switzerland

The adoption of common transnational approaches and protocols for water quality monitoring in the Alpine region is one of the main aim of the Eco-AlpsWater project. In this area, most of the countries adopt national/regional methods compliant to the WFD. Even though, many differences among countries have been pointed out. Moreover, Switzerland, as a non-EU country, applies methods based on the Swiss Water Protection Ordinance (WPO). Therefore, the harmonization of the approaches for water quality assessment across the Alpine region is recommended. The EAW project evaluated how and where innovative approaches based on eDNA and HTS technologies could bridge the gaps and weakness of traditional methods, to develop a next generation monitoring approach shared across the countries in the Alpine space.

The six key lakes (Bled, Bourget, Garda, Lugano, Mondsee and Starnberg) and the five key rivers (Adige, Drome, Soča, Steyr and Wertach), selected as pilot sites in the Eco-AlpsWater project, were used for a comparison of methods adopted in the determination of the ecological status on the basis of biological quality elements (phytoplankton, phytobenthos, and fish). This wide survey in the alpine region highlighted the weakness and potential implementation of approaches intra and inter countries, in the context of previous intercalibration processes.

Requirements of the EU-WFD and CH-WPO

The countries in the Alpine region, belonging to the EU, adopt regional/national methods based on the WFD. In the last years, intercalibration processes (IC) have been carried out, which allowed a harmonization of approaches among countries (e.g. phytoplankton). Despite different indexes are used in each country, the assessment of the ecological status is reported in five quality classes (High, Good, Moderate, Poor, Bad) for a correct comparison of the waterbodies (https://ec.europa.eu/environment/pubs/pdf/factsheets/water-framework-directive.pdf).

In Switzerland, the Swiss Modular Stepwise Procedure has been developed, which involve status classes comparable with the system of ecological classes defined in the WFD. Based on the WPO, methods for the determination of the ecological status using the biological quality elements have been designed by the Federal Office for the Environment (FOEN). However, these protocols represent guidelines for Cantons, which have the legislative power to decide about the terms of their application in their own territory. At the moment, only methods for diatoms and fish in rivers have been published. Nevertheless, over the years each Canton has applied also internal protocols (e.g. phytoplankton) and the standardization at Federal level is under development. Moreover, the transboundary waters (Switzerland-EU country) are under the control of International commissions (i.e. CIPAIS, CIPEL, IGKB) which have specific goals and involve different types of quality indicators,

sometimes in contrast to the WFD and WPO laws. As example, in Lake Lugano, at the border between Switzerland and Italy, the reference conditions vary among the regulations involved: in the WFD, good ecological status should be achieved or maintained; in the WPO, near-natural ecological status should be achieved, with a diversity and abundance of species specific to unpolluted or low-polluted waters; in CIPAIS the reference status is represented by mesotrophic conditions. Therefore, different approaches for water quality assessment adopted in Switzerland are a clear example of the needs of homogenization.

Besides Switzerland, the survey carried out first in key lakes and rivers and then extended to the whole Alpine region, also highlighted differences in the application of WFD among the EU-countries. In a few countries, standardization of methods for some BQE are still under development or not yet started. The main differences regard phytobenthos, which is being implemented. A summary of the application of BQE methods for water quality assessment in the Alpine region (Table 1). Similarities and differences in the Alpine region (Table 2).

Table 1. A summary of the application of BQE methods for water quality assessment in the Alpine region (phytoplankton, benthic diatoms and microalgae, and fish.

Alpine region BQE Methods		Phytoplankton	Benthic Diatoms	Benthic microalge (excluding diatoms)	Fish
Austria	**Lakes**	yes	no	yes	yes
	Rivers		yes	yes	yes
France	**Lakes**	yes	no*	no	yes
	Rivers		yes	no	yes
Italy	**Lakes**	yes	yes	no	yes
	Rivers		yes	yes †	yes
Germany	**Lakes**	yes	yes	no	yes
	Rivers		yes	no	yes
Slovenia	**Lakes**	yes	yes	yes ††	yes
	Rivers		yes	yes ††	yes
Switzerland	**Lakes**	yes	no	no	no
	Rivers		yes	no	yes

* method under development

° method available at Canton level

† benthic microalgae are considered in monitoring river macrophytes when they form macroscopic aggregates.

†† only limited to specific algal groups

The fine comparison of the features of BQE methods adopted in the key lakes and rivers showed a good consistency, which support the feasibility of homogenization. As reported in the table above, the method adopted for monitoring phytoplankton in lakes is almost entirely shared among the countries, except for the sampling frequency. As discussed before, the monitoring of phytobenthos is being implemented, and some countries applied this BQE exclusively on rivers. Moreover, in some countries filamentous green algae are also included (e.g. Slovenia), while in most of them only diatoms are considered. Other two main aspects vary in the monitoring of phytobenthos: the number of sampling stations and the sampling frequency. These features vary also in the monitoring of fish, suggesting a potential improvement of the traditional methods.

Table 2. Similarities and differences in the Alpine region monitoring of lakes and rivers for phytoplankton, phytobenthos and fish.

Biological elements		**Similarities**	**Differences**
Phytoplankton	Lakes	Sampling point (max. depth) Sampling depth (epilimnion/euphotic depth) Biovolume (Utermöhl) and Chlorophyll-a (ISO) Species level identification	Sampling frequency
Phytobenthos	Lakes	Sampling period Substrate types Habitat Species/genus level identification	Biological community (phytobenthos other than diatoms) Number of sampling stations Sampling frequency
	Rivers	Sampling period Sampling site features Substrate types Habitat Species/genus level identification	Biological community (phytobenthos other than diatoms) Number of sampling stations Sampling frequency
Fish	Lakes	Sampling period Sampling strategies Species level identification	Number of sampling stations (surface/depth)
	Rivers	Sampling period Sampling strategies Habitat Species level identification	Number of sampling stations Sampling frequency

The differences highlighted support the use of HTS for the improvement and homogenization of approaches across the Alpine region. Besides the potentiality of HTS in the study of biodiversity, indeed, metagenomics could help for example in increasing the spatio-temporal and taxonomic coverage in monitoring, reducing the timing and cost of the analysis. The innovative protocols were tailored for the WFD/WPO traditional monitoring, in order to explore thoroughly the biodiversity of the aquatic ecosystems and provide a more complete taxa inventory. This process was possible thanks to the cooperation with observers and stakeholders, which provided their feedback for the tuning of the approaches.

The next generation monitoring approaches

The integration of innovative methods based on eDNA analyses and NGS technologies in the next monitoring approaches for the assessment of biodiversity and ecological status of waterbodies was positively embraced by Stakeholders and Policymakers. They identified potential advantages and opportunities:

- More information obtained through a single sampling
- Increase of the spatio-temporal coverage in monitoring
- Possibility of sampling hard to reach locations and study complex environments
- Investigation of a larger part of biodiversity, including biological groups whose taxonomy is complex and time-consuming, and taxa which are not monitored yet, thus to obtain a more taxonomically complete inventory and answer additional ecological questions
- Detection of non-native and native (e.g. rare) species
- Detection of pathogens and vectors
- Less invasiveness (e.g. for fish and in vulnerable ecosystems)
- Faster, cheaper (possible analysis of numerous samples)

All these favourable aspects support the use of eDNA approach as a complementary tool to existing methods for ecological status assessment and water management, and as a way to facilitate the harmonization EU and CH approaches. To reach this goal, national strategies are required, including a standardization of eDNA approaches, the establishment of exhaustive reference databases, and the acquisition of new competences by environmental agencies in charge of water quality monitoring. In conclusion, the transnational innovative approach represents a strategic element aiming to enhance the protection, the conservation and the ecological connectivity of Alpine Space ecosystems, and has the potential to be applied even at a wider European scale.

VII. FAQ - general perspective

We have collected questions (FAQ) about the metabarcoding approach during our stakeholder meetings. Here we provide more general answers, but you can also find answers from the scientific perspective at our webpage (FAQ Catalogue).

Why several primers are used in the Eco-AlpsWater metabarcoding approach?

We need several primers because different target DNA regions are used to distinguish organisms. The phylogenetic relationships of the biological target organisms such as bacteria, microalgae and fish are not close.

What causes the inability to achieve a fine taxonomic resolution (at species level) with the Eco-AlpsWater metabarcoding approach for microalgae?

Microalgae belong to very different phyla of the evolution tree. Therefore, we used generalist markers to detect the global microbial assemblages. These markers recovered a lot of hidden species, but conversely they failed to detect circumscribed groups of traditional indicator species. Moreover, several species that have been described in literature are not yet represented in the molecular reference databases.

How to compare taxa inventories with a mix of species, genera and orders?

Both HTS and light microscopy methods can detect the same genus, but different species, or stop at just the genus. The Eco-AlpsWater taxa analysis tool delivers match tables on genus or on species separately for cyanobacteria and eukaryotes.

How to interpret the name of the species listed under several DNA sequences in the metabarcoding outputs?

The number of DNA sequences (amplicon sequence variants, ASVs, or olygotypes), which belong to one unique species (taxon), is an indicator of the intra-specific (intra-taxon) genetic diversity. The Eco-AlpsWater taxa analysis tool brings all sequences together, which belong to the same taxon and aggregate the result in one “present” record.

How to select my target taxa? I see metabarcoding output lists with many taxon names, which I never heard before.

Taxon names in metabarcoding lists are up-to-date, and thus, many biological names may be new for the user. Users are familiar only with those of the traditional monitoring, with taxa names frequently synonymous with the updated taxa names and grouped in an old-fashioned systematic. Common codes

and taxa names for phytoplankton and benthic diatoms are used in the Eco-AlpsWater taxa analysis tool to compare the lists.

How long does eDNA stay in water? Are the eDNA of different organisms differently resistant? How long can dead organisms excrete eDNA?

By the term "environmental DNA" (eDNA) we mean the entire hereditary material of all organisms that are (or have been) present in the environment. This genetic material can be derived directly from the cells of microorganisms that are sampled along with water (e.g. microscopic algae or bacteria). In larger organisms (e.g. fish or humans), it is transferred to the environment through body secretions, dead skin, hair and can be stored in the form of DNA molecules in the aquatic environment for several days or even weeks. The stability of DNA in the aquatic environment depends on the conditions in the environment (temperature, pH, oxygen, light, and other substances in the water). If DNA is trapped in sediments at the bottom of water bodies, it can stay there for years or decades; in some cases even millennia, which opens the door to paleoecological research.

Why did you exclude macrophytes and benthic invertebrates from all biological parameters?

Due to the financial constraints of the project, these two, otherwise extremely important biological elements, were not included.

Can the 18S rRNA gene detect Euglena and other euglenids?

In general, no primer pair can target all protists equally well. The specific primer pair that was chosen to amplify the 18S rRNA gene was unable to detect euglenids in water samples. Instead, we are using information from the chloroplast 16S rRNA gene to detect this group.

Which taxonomic level is achievable with which marker?

This depends on the groups of organisms and gene regions selected, and the completeness of reference databases. While the genetic markers for diatoms are highly curated and specialized, the markers for bacteria and phytoplankton are more general. Therefore, the first marker (rbcL) can achieve more detailed classifications, while the second group of markers (16S and 18S rRNA) detect mainly genera or higher taxonomic ranks. To identify organisms at species level, very specific primers in connection to specific taxonomic reference databases should be used. To fully exploit the information present in the sequences, further phylogenetic analyses should also be performed to support and complement the classifications obtained from bioinformatics pipelines or BLAST analyses.

Which taxa were detected with each marker in the Eco-AlpsWater data set?

The full list of taxa and genotypes detected using HTS within the Eco-AlpsWater data set are given in the HTS taxonomy list of each marker. When focusing on species or genera of the bio-components

(connected to common Eco-AlpsWater codes), we detected 88 cyanobacteria, 582 phytoplankton (excl. Cyanobacteria), 226 diatoms and 54 fish taxa, many of them with several genotypes. The lists are included in the Eco-AlpsWater taxa analysis tool.

Which specific logistic requirements are necessary when sampling eDNA from plankton samples?

The Eco-AlpsWater project recommends sterile encapsulated filters (Sterivex), DNA-free bottles and gloves to reduce contamination, but see all details in our YouTube video and the sampling protocols. Deep-frozen storage of filters until DNA extraction for up to 9 month were successful in our test.

Who helps me to interpret the HTS results when unknown taxa were recorded by HTS?

Additional analyses, such as BLAST queries, can provide deeper understanding of closely related taxonomic taxa and groups. Improved genetic reference databases, which are curated for a specific taxonomic group and/or eco-regions, can increase the accuracy of species classifications.

What is a BLAST analysis carried out for cyanobacteria?

For cyanobacteria and other biological taxonomic groups, automated taxa assignment was improved by using reference sequences from relevant taxonomic literature, i.e. using (morphologically described) isolates (strains) and manual blasting against the obtained cyanobacteria ASVs. BLASTn-induced changes in the taxon name for selected ASVs in 16S were marked in the Eco-AlpsWater taxa analysis tool.

How does the VigiDNA® method for fish eDNA sampling work?

VigiDNA® is the product name of the filter cartridges, used in the Eco-AlpsWater project to analyse fish biodiversity in Alpine waters. These closed, encapsulated filter cartridges (VigiDNA®, Spygen®) are used to filter large volumes of water (up to 30 litres or more) collected along lake shores or in the middle of rivers. After filtration, the filter cartridge is filled with a preservation buffer and stored at room temperature until DNA extraction.

Which river types are suited to be analyzed using the VigiDNA® system?

Basically, this system is suitable for any type of river and allows filtering up to 30 litres of water with a single cartridge. However, it might be challenging in rivers with increased particle load in the water as fine sediment can cause the filter to clog before the desired 30 litres have been filtered. Therefore, it is advisable to adjust sampling accordingly, e.g. samples should not be collected during, or shortly after, flood events.

Which primer pair is used for the fish eDNA analysis?

For the sequencing of fish eDNA samples, the MiFish-U primers were used. This primer pair is regularly used for fish metabarcoding studies.

Can eDNA detections be assigned to a specific river section?

This depends on the sampling design. As the eDNA is constantly transported downstream, only fish species occurring upstream of the sampling point may be detected in the follow-up analysis.

Can the number of individuals be deduced from the frequency of the detected sequences per species?

No, so far it is not possible to make precise statements on absolute abundances of different fish species based on the number of detected reads.

Which different approaches for fish eDNA assessment were used in the Eco-AlpsWater project?

In total, 3 different approaches were used. The VigiDNA approach, where 30 litres of water are filtered through an encapsulated filter cartridge. The Sterivex® (0.45 µm porosity) point sampling approach, where 2 litres of water where collected at the start, in the middle and at the end of each VigiDNA lakeshore transect. And a GFC (glass fiber filter, 1.2 µm nominal porosity) point sampling approach, where 5 litres of water were collected at traditional sampling sites.

Join our Eco-AlpsWater Alpine Network at:
https://www.alpine-space.org/projects/eco-alpswater/en/project-results/Eco-AlpsWater-alpine-network

and follow our Eco-AlpsWater activities further!

This brochure was created as part of the Eco-AlpsWater project, which is partly funded by the European Union from the European Regional Development Fund (support from the EU: € 1,447,666.54). The project was implemented within the INTERREG Alpine Transnational Cooperation Program for the period 2014-2020.