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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Eco-AlpsWater Toolbox for the implementation of innovative monitoring approaches

Interreg Alpine Space - Eco-AlpsWater project – WP3

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ABSTRACT

This document includes the description of all the steps required to complement the traditional monitoring procedures with methods based on the use of the environmental DNA (eDNA) coupled with High Throughput Sequencing (HTS) to assess the biodiversity of macro and microorganisms used as indicators within the European Water Framework Directive (WFD, 2000/60/EC). All the procedures were developed to be efficiently integrated within the conventional biomonitoring plans. In parallel with the development of the new protocols, a survey covering the whole Alpine region was realised to verify the gaps and potential implementation of conventional approaches intra and inter countries, and to provide tools and guidelines a homogeneous and coherent integration of HTS-based approaches. The number of protocols produced is very high. At the first glance it may not be an immediate task to identify and associate the protocols to the specific technical steps in the general procedure required to complete an HTS analysis. This document is intended to be a logic guide, providing help in the description, significance, and chronological use of these steps, and in the choice of the suitable protocols (Annex 1).

Eco-AlpsWater Toolbox for the implementation of innovative monitoring approaches.

❖ Introduction

This document includes the description of all the steps required to complement the traditional monitoring procedures with methods based on the use of the environmental DNA (eDNA) coupled with High Throughput Sequencing (HTS) to describe the biodiversity of macro and microorganisms used as indicators within the European Water Framework Directive (WFD, 2000/60/EC). A number of intercalibration processes have been implemented to ensure a coherent implementation of this Directive.

Within the Eco-AlpsWater (EAW) project, a survey covering the whole Alpine region was used to verify the gaps and potential implementation of approaches intra and inter countries, and to provide tools and guidelines for the harmonic integration of HTS-based approaches.

One of the goals of INTERREG Eco-AlpsWater project was to evaluate and validate up-to-date experimental protocols to provide useful guidelines for stakeholders and governmental agencies. This task allowed to create an active network, providing flexible instruments to be applied also in other contexts.

The pre-sampling activities and the resulting draft sampling protocols were discussed with Observers and Stakeholders to get feedback for the implementation of the eDNA approach. Observers were invited to project meetings at Milano, Italy and Mondsee, Austria in addition to local meetings organized in each country. In some countries also knowledge Stakeholders (e.g., local or regional public authorities) were invited to local meetings. Their feedback and expectations, together with the inputs of Project Partners (PP), provided important elements to support the strategy to be adopted in the project for the integration of traditional and innovative eDNA methods in the evaluation of water quality. Each Project Partner provided documents on the traditional national monitoring methods with detailed information regarding sampling and

analysis of biological quality elements (BQE), according to both Water Framework Directive (2000/60/EC-EU WFD) and Water Protection Ordinance-WPO 1998. The protocol workflow was designed to be consistent with traditional methods in the Alpine region and was implemented after feedback of PPs, collected from Observers and Stakeholders during local meetings.

Different technical aspects were resumed and discussed for each Biological Quality Element: sampling devices for water samples, depth of integrated samples according to euphotic zone and historical datasets, mandatory and recommended environmental data, consistent use of site and stations historically monitored, period of sampling, heterogeneity of habitats to cover biodiversity, list of species and eDNA approach limits.

However, the common goal of fine-tuning the eDNA sampling plan with traditional monitoring activities was a consistent mutual integration, providing sustainable and easy-to-implement protocols, valid for each country. Therefore, innovative protocols should consider the local site condition and historical datasets for each pilot site (e.g., sampling depth, sampling period, sampling area, environmental variables).

❖ Monitoring approaches presently adopted in the Alpine Space region and EU, and experiences learned from the implementation of pilot actions within Eco-AlpsWater.

Since 2000 the European Union has been actively engaged in the protection and enhancement of aquatic ecosystems: freshwater biomonitoring promoted by the implementation of the EU Water Framework Directive (WFD, 2000/60/EC) allows the evaluation and improvement of their ecological quality status. However, the classical biomonitoring practices require good taxonomic expertise for the morphological identification of specimens and tend to be non sensitive regarding low abundant or elusive species, thus providing partial estimates for entire communities (Deiner et al. 2017). In the Alpine region, procedures for freshwater biomonitoring are also used by the Swiss Federal Office for the Environment (FOEN) and

recommended to cantonal administrations. It was therefore paramount to identify weaknesses and gaps in the present monitoring approaches used in different regions to evaluate the possible improvements and suggest a harmonization of the current survey methods. The output of the Deliverable D.T2.1.2 consisted in a collection of methods adopted in the Alpine region for the assessment of the ecological status of waters, using the biological quality elements selected in the Eco-AlpsWater project (phytoplankton, benthic diatoms, and fish). The outputs of Deliverable D.T2.1.1 were integrated with procedures suggested by the Swiss Federal Office for the Environment (FOEN) and recommended to cantonal administrations, and with results from a previous wider survey at the European scale, the WISER project (2012). Moreover, to implement information collected in the WISER database, some PPs provided an updated version of the methods used for the evaluation of BQE in the Alpine region. Below, we report a summary of BQE methods adopted in the Alpine space available for each country (Table 1), applied in key lakes and rivers of the Eco-AlpsWater project (EAW), retrieved from WISER database (WD), the Phytoplankton Index for Lakes (IPLAC), provided by PPs (PP) or recommended by FOEN (FOE). A detailed description is reported in the Deliverable D.T2.1.2 (EAW website).

Table 1. Summary of BQE methods adopted in the Alpine space available for each country.

Alpine region BQE Methods		Phytoplankton			Diatoms (Phytobenthos)			Fish		
Austria	Lakes	EAW	WD					EAW	WD	
	Rivers				EAW	WD		EAW	WD	
France	Lakes	EAW	IPLAC		EAW			EAW		
	Rivers				EAW	WD		EAW	WD	
Italy	Lakes	EAW	WD	PP	EAW		PP	EAW	WD	
	Rivers				EAW		PP	EAW		
Germany	Lakes	EAW	WD		EAW	WD		EAW		
	Rivers				EAW	WD		EAW	WD	
Slovenia	Lakes	EAW	WD		EAW	WD		EAW	WD	
	Rivers				EAW	WD		EAW		
Switzerland	Lakes	EAW								
	Rivers					FOE			FOE	

- **Transferable, shared, and approved tools and monitoring approaches for the assessment of ecological status and biodiversity of lakes and rivers in the Alpine Space region and Europe. Groundwork for the next monitoring approaches in lakes and rivers.**

The methods required for the analysis of eDNA coupled with HTS are formalised in several guidelines that the EAW consortium made freely available to stakeholders and governmental agencies which can follow the different steps described to complement the current traditional methods with these up-to-date approaches.

The species list obtained for the different organism groups targeted by using the eDNA metabarcoding approaches have been uploaded in an Access database in which all the data and metadata for each sample has been recorded (EAW Taxa Analysis Tool). A list of ready to use queries have been created to extract selected information allowing easy access to data and metadata (Fig. 1).

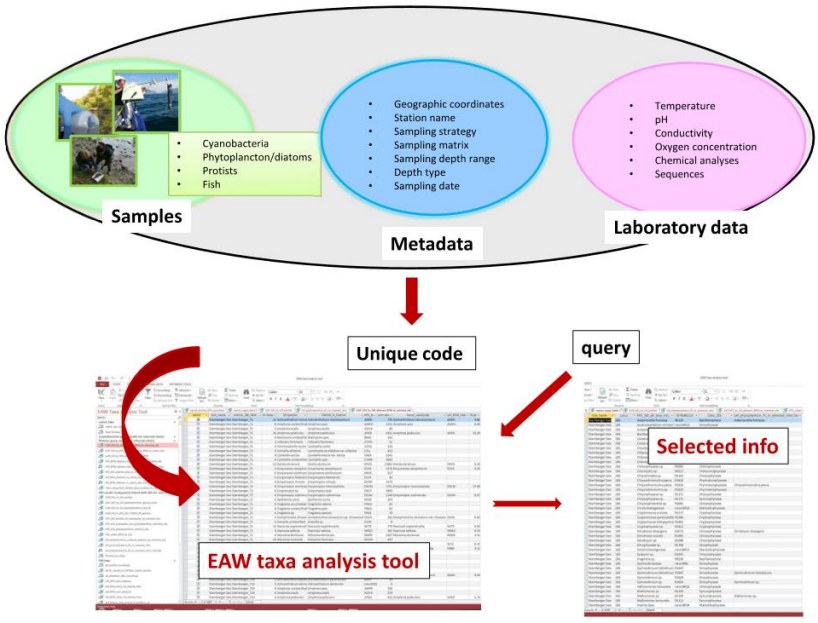

Figure 1: Samples management scheme

❖ Conceptual scheme.

The INTERREG Eco-AlpsWater (EAW) project focuses on the use of eDNA metabarcoding approach to assess the biodiversity and ecological status of lakes and rivers in the Alpine region. Different biological groups are targeted within the EAW project, including bacteria (cyanobacteria), protists (microalgae), and fish.

eDNA assessment of aquatic biodiversity from environmental samples involves different steps (i.e., eDNA workflow): sample collection, DNA extraction, amplification of barcode regions with selected primers, sequencing amplicons, bioinformatic treatment and downstream ecological indices/metrics and statistical analysis. Multiple parameters may affect the final molecular species inventory all along the DNA workflow HTS; multiple choices exist and different methods can be applied, therefore guidelines for metabarcoding need to be defined to allow its standardization for biomonitoring purposes for each biological target. For example, according to Piggott (2016), in the metabarcoding of fish communities the choice of sampling and extraction method and PCR strategy, rather than amplicon size and marker region, had the biggest effect on detection probability and PCR replication. However, it is well known that primers used in the initial amplification of barcode sequences can introduce significant biases (Elbrecht and Leese 2015) and the accuracy in delimiting species strongly depends on the DNA marker selected (Rach et al. 2017).

The samples are collected by following specific protocols for each different kind of basin (lake or river) and biological matrix (water or biofilm). The laboratory procedures (DNA extraction, library preparation and HTS) allow to obtain a vast number of sequences that need to be further processed by using bioinformatic pipelines to obtain lists of identified species. The list of species is then compared with the lists of species obtained from the traditional monitoring approach and, in case of discrepancies, the respective sequences are further inspected.

The total list of species (traditional plus HTS) is used to compute metrics and indices and obtain the Ecological Quality Ratio for each ecosystem: these values are compared with historical data to evaluate how HTS methods can improve the evaluation of the ecosystem status of the surveyed habitats.

All the steps required to perform an integrated survey of biodiversity by coupling the traditional and HTS approaches and to provide recommendations for policy planning and management are resumed in Fig. 2.

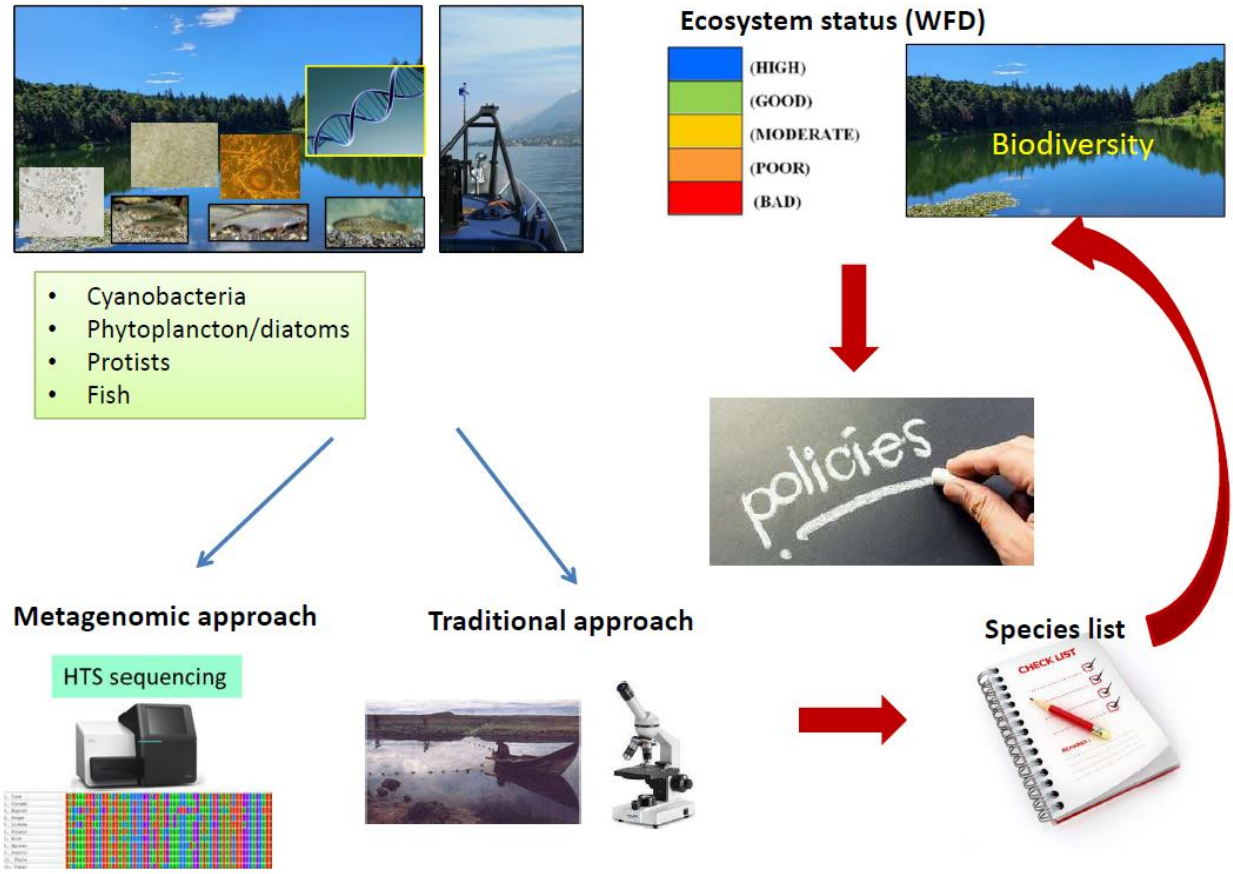

Figure 2: Conceptual scheme of Ecosystem status assessment coupling traditional and HTS approaches.

❖ Formalised protocols and guidelines

One of the aims of the EAW project was to test and evaluate the different procedures required for eDNA metabarcoding assessment of freshwater biodiversity. These exercises allowed to obtain accurate and detailed protocols for each step of the analysis process, i.e., from sampling to bioinformatic analyses of HTS sequences (Fig. 3 and Annex 1). A synthetic and systematic description of these protocols have been also included in the e-booklet 2 https://www.alpine-space.org/projects/eco-alpswater/publications-booklets/2-en--technical-guidelines.pdf

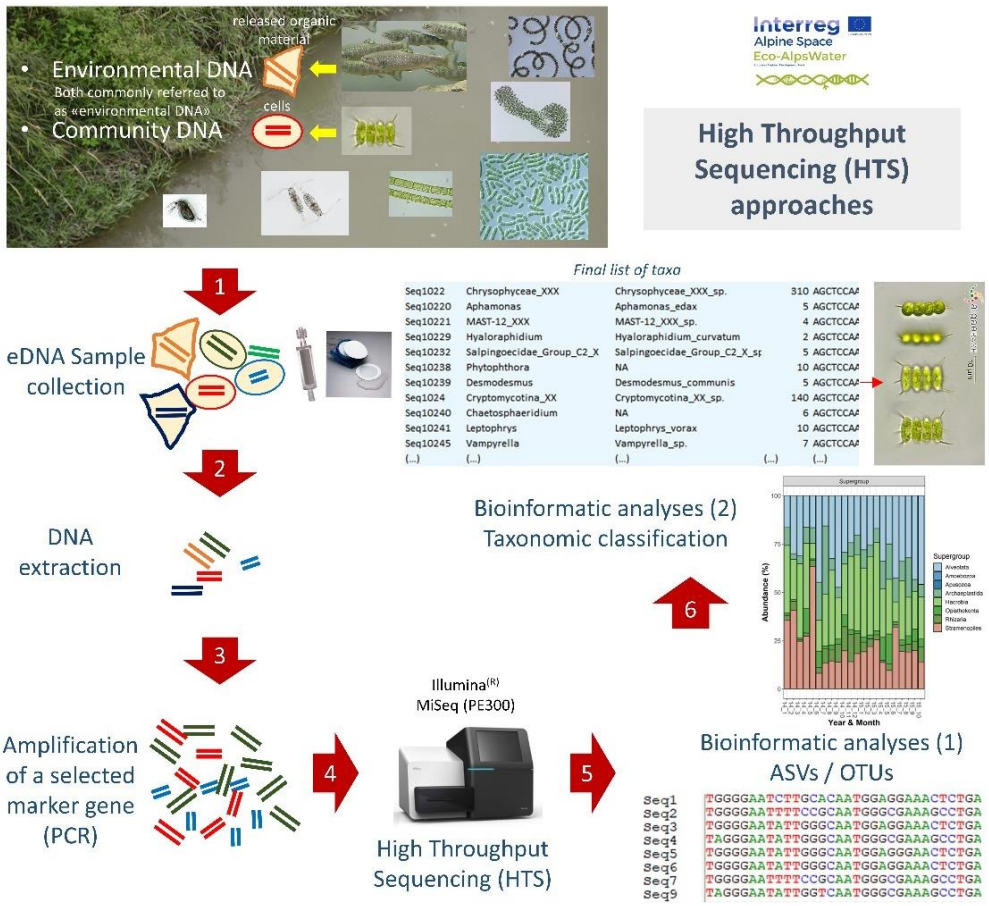

Figure 3. Synthetic representation of the major common steps applied in the analysis of eDNA in freshwater ecosystems (lakes and rivers). The figure includes an example of amplification, sequencing, and classification of protist organisms (red, blue, and dark green DNA sequences), but the steps are the same for other aquatic organisms. However, each biological element requires specific adaptations of the procedures in all six steps of HTS.

Sampling procedures

EAW consortium has been actively involved in the identification of the best methods for the implementation of the eDNA HTS approaches. Different sampling procedures have been used for biofilm and water (Step 1 in Fig. 3).

For plankton sampling, the collection strategy is like that used for classical phytoplankton survey focusing on the euphotic zone, however the procedure for filtration and preservation is adapted for eDNA samples. The sampling location for the eDNA approach is the same as for traditional monitoring of phytoplankton: the deepest location or the midpoint of the lake (the historical sampling point on which long term records are based). The sampling strategy follows the integrative method applied for phytoplankton which require the use of Integrating Water Samplers (e.g., bottle IWS; a pool of discrete samples taken with a Niskin bottle; see standard procedures CEN).

Samples of water are filtered on-site to ease the sampling activities (avoiding the transport of large volumes of water, especially when sampling campaigns last for days or weeks) and avoid DNA degradation. Nevertheless, the filtration in the lab offers better conditions in terms of sterility and ease of handling. Water is filtered by using enclosed (encapsulated) filters that reduce the risk of contamination. Specifically, 0.22 µm filters are used for bacteria and plankton analyses.

For biofilm sampling two different methods are used, one adapted for lakes and one for rivers. This field protocol is based on routine methods used for biofilm sampling and agrees with:

- CEN 2014. Water quality - NF EN 13946 - Guidance for the routine sampling and preparation of benthic diatoms from rivers and lakes. Afnor, 1-23.

- CEN 2018. Water quality - FprCEN/TR 17245 - Technical report for the routine sampling of benthic diatoms from rivers and lakes adapted for metabarcoding analyses. CEN, 1-8.

Phytobenthic communities' composition changes along seasons. In large lakes, the major variables explaining these temporal changes are nutrients (especially phosphorus). Moreover, the heterogeneity between the communities along the shoreline vary from a season to another, and is more distinct in summer (e.g. Lake Geneva in Rimet et al. 2015). For this reason,

samplings must be carried out during summer. In general, sampling sites free of submerged (and emerged) macrophytes should be selected to exclude shadowing effects on phytobenthic growth which are most intense during summer. If wind induced strong waves during several days and scoured the biofilms (e.g. storms, days with continuous strong winds), then it is advised to wait for 2-3 weeks before collecting the samples, to allow biofilms to restructure. Water level fluctuations also have a significant effect on biofilms community composition. Water level should be stabilized at least over the past 3 weeks before sampling.

For each sampling site at least 5 stones (it can be more, depending on stones sizes and biofilms amounts), for a total brushed surface of at least 100 cm^2, are collected. Stones are taken at 50 cm depth from the minimal water level (recorded annual data) in an area of 100 m^2. The stones are brushed with a new sterile brush and the biofilm is mixed with sterile water: a subsample of this mixture is used for eDNA analyses, the remaining mixture is added with absolute ethanol and stored for traditional microscopic analyses.

For river biofilm collection, the sampling is usually carried out during low flow season in the framework of national river monitoring networks, optimally during the natural low-water period of the respective water body under clear water conditions (i.e., summer in Europe). Flood events must be taken into consideration: if low intensity hydrological events appear (low intensity floods, floods of a few days duration, aerial exposure of a few days), it is recommended to wait a few days before taking samples. After intense hydrological events (lasting floods, floods causing a reworking of the supports), it is recommended to wait 3-4 weeks before taking the samples. In the case of a site subjected to strong artificial variations of the flow (after dams for instance), the artificial hydrological regime is assimilated to a stabilized flow.

The sampling protocols for fish biodiversity assessment through eDNA metabarcoding consisted of three different approaches, resumed below.

1) First, a large volume of water (ca. 30 L, integrative approach) was collected along the lake shore or within the main river course to be representative of the water body and increase the possibility of collecting DNA of rare fish. The collected water was then filtered by using 0.45 µm enclosed filters with a filtering capacity of 30 L. This method allowed to continuously

collect water along transects or integrative depth strata. A preservation buffer is added to the cartridge, which is then stored at room temperature until extraction. However, the cartridges filled with the preservation buffer should not be stored for too long, it is advised to extract the DNA within one month after sampling. 2) The second approach (used to compare results with the first one) was based on the point sampling collection of 2 L of water, filtered through a Sterivex™ 0.45 µm filter cartridge (encapsulated filter). 3) In the third approach, 5 L water samples were taken from the same depths and sites (gill net locations & electrofishing stretches) used for the traditional fish status assessment, and filtered through glass fiber filters (GFC, nominal porosity 1.2 µm); this approach is not only useful to obtain taxonomic inventories of fish in a water body, but also allows to identify spatial distribution patterns of the detected species.

Sampling for fish eDNA should ideally be carried out before the traditional fish monitoring methods are deployed. Collecting eDNA samples one week before the start of traditional investigations would therefore ensure that no "foreign" fish DNA is detected and that only local fish biodiversity, present at the date of sampling, is assessed.

DNA extraction

DNA extraction (Step 2 in Fig. 3) is a critical step for obtaining relevant results; because many plankton species have tough cell walls, methods for cell lysis and DNA isolation need to be efficient to allow unbiased nucleic acid retrieval.

For the Eco-AlpsWater project, plankton sampled in lakes was filtered through encapsulated Sterivex cartridges (Sterivex™ GP 0.22 µm) and stored at -20 °C. The methodology chosen for DNA extraction is therefore adapted to the type of material/filter used for plankton collection (i.e., Sterivex cartridge). The DNeasy® PowerWater Sterivex Kit (QIAGEN) with specific modifications adapted to plankton DNA extraction has been used.

The choice of the methodology for biofilms DNA extraction is based on previous studies and on the work done by Vasselon et al. (2017). For the Eco-AlpsWater project, after sampling in lakes or rivers, biofilms are stored in ethanol (absolute) in 50 mL falcon tubes 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 chosen for biofilm extraction has been used in several recent studies (e.g., Vasselon et al 2017ab, 2018) focussed on the application of diatoms metabarcoding; this method is based on a protocol adapted from the NucleoSpin® Soil kit (MACHEREY-NAGEL) with specific modifications for biofilm DNA extraction.

Three different extraction methodologies have been used for fish DNA samples. The choice of the methodology is based on previous studies and on the study of Pont et al. (2018), with some adaptations for the Eco-AlpsWater project. 1) The DNA extraction protocol for the fish eDNA samples collected with the VigiDNA® 0.45 µm capsule (or alternatively Envirochek HV 1 µm) is based on the NucleoSpin® Soil kit (MACHEREY-NAGEL) with specific modifications according to the detailed protocol presented by Pont et al. (2018) and the tests done in the context of the Eco-AlpsWater project; as anticipated in the previous section, it is strongly advised to extract the DNA within one month after sampling. 2) For Sterivex filters the extraction protocol of Pont et al. (2018) has been modified to adapt to these different cartridges that are used for point sampling. A recent revision of this method has been reported in Vautier et al. (2021). 3) For the DNA extraction of the GFC filters, the Dneasy® PowerWater Kit (QIAGEN) was used and the extraction was done according to the manufacturers protocol.

Library preparation for MiSeq Illumina sequencing.

Different approaches have been used for the laboratory procedures depending on the target organisms and genetic markers used (Step 3 and 4 in Fig. 3).

For bacterioplankton eDNA, the protocols adopted the specific primer set 341F (5'-CCTACGGGNGGCWGCAG-3', Klindworth et al. 2013) and 850Rmod (5'-GACTACNVGGGTWTCTAATCC-3', Klindworth et al. 2013; Apprill et al. 2015) with overhang Illumina adapters. This couple of primers amplifies the total genomic DNA by targeting a ~ 460-bp fragment of the 16S rRNA variable regions V3–V4. PCR amplification was carried out using 25 µL reactions with 1 µM of each primer. In the successive step, dual indices and Illumina sequencing adapters Nextera XT Index Primer v2 (Illumina) were attached

by 7 cycles of PCR (16S Metagenomic Sequencing Library Preparation, Illumina).

Eukaryotic microplankton eDNA analyses were performed by amplifying a 380 bp fragment of the 18S rRNA gene variable region V4 using the specific primer set TAReuk454FWD1 (5'-CCAGCASCYGCGGTAATTCC-3'; Stoeck et al. 2010) and TAReukREV3_modified (5'-ACTTTCGTTCTTGATYRATGA-3'; Stoeck et al. 2010; Piredda et al. 2017) with overhang Illumina adapters. PCR amplification and library construction were performed as described in Salmaso et al. 2018.

For benthic diatoms, the *rbcL* plastid gene has proved to be an suitable marker for taxonomic identification (Vasselon et al. 2017a, b) and a well- curated barcode reference library is already available to assign species names to *rbcL* sequences (R- Syst::diatom, Rimet et al. 2016). PCR amplifications are performed on *rbcL* gene targeting a 312 bp barcode. For the amplification of this region, the primer pair Diat_rbcL_708F (Stoof-Leichsenring et al. 2012) and R3 (Bruder and Medlin 2007) has been slightly modified. Using an alignment of 1602 *rbcL* reference sequences from 638 diatom species, the degeneracy of the primers was increased to amplify a broader diversity of diatoms. A two-step PCR amplification procedure has been used. For each DNA sample, the first PCR amplifications are performed in triplicate in a final volume of 25 µL. PCR1 amplicons are purified and used as templates in the PCR2 which used Illumina-tailed primers targeting the half of P5 and P7 sequences. Finally, all generated PCR2 amplicons are dual indexed and pooled into a single tube. Final pool is sequenced on an Illumina MiSeq platform using the V3 paired-end sequencing kit (250 bp × 2).

For fish eDNA analyses custom tagged primers are used for the first round of PCR which represent the first step for the preparation of double tagged HTS libraries. The custom tagged primers were developed and validated using the EDITTAG software (Faircloth & Glenn, 2012) and consist of the MiSeq sequencing primers, a seven base-pair tag and the fish specific MiFish-U primers (forward: 5'-GTCGGTAAAACTCGTGCCAGC-3', reverse: 5'-CATAGTGGGGTATCTAATCCCAGTTTG-3' Miya et al. 2015). During the second round of PCR, standard Illumina tagged primers are used to add the second set of tags and the MiSeq flow cell adaptors to the PCR product from the first round of amplification.

Bioinformatic analyses

The biological molecular methods based on metabarcoding and HTS produce a huge amount of data that need advanced bioinformatic methods to be analysed and obtain manageable outputs (Step 5 and 6 in Fig. 3). Different bioinformatic protocols are currently used by the scientific community for the analysis of metabarcoding data. For each marker gene, protocols were chosen and adapted from dedicated pipelines that provided the best results in previous studies. Bioinformatic pipelines and tools developed in these recent years include, among the others, QIIME2 (Estaki et al. 2020), OBITools (Taberlet et al. 2018), Vsearch (Rognes et al. 2016) or DADA2 (Callahan et al. 2016). These bioinformatic tools typically perform bioinformatic analyses such as data denoising, sequence alignment, clustering into Molecular Operational Taxonomic Units (MOTUs) or amplicon sequence variants (ASVs), taxonomic assignment and produce a MOTU/ASV-by-sample matrix. This matrix can then be used for further statistical analyses and data processing.

A pipeline adapted for the identification of amplicon sequence variants (ASVs) and implemented in the DADA2 protocol (Callahan et al. 2016) has been used for the identification of bacteria and cyanobacteria within the Eco-AlpsWater project. In general, different approaches can be adopted for the analysis of HTS 16S rDNA reads. These can be based on the identification of OTUs built at specific levels of identity (generally 97%) (Edgar, 2018) or, as more recently proposed, on the identification of individual variants using oligotyping approaches (Eren et al. 2013, 2015) and denoising methods, which identify amplicon sequence variants, ASVs, also known as exact sequence variants, ESVs. As for the latter approach (ASVs), a number of methods have been proposed, including DADA2 (Callahan et al. 2016), DEBLUR (Amir et al. 2017), UNOISE 2 and 3 (Edgar, 2016); a few of them have been implemented in QIIME2 (DADA2 and DEBLUR; Bolyen et al. 2019) or adapted in VSEARCH (such as UNOISE). The effectiveness of denoising approaches compared to OTUs methods has been substantiated in a number of investigations using bacterial (e.g., Prodan et al. 2020, and references therein) and fungal (Pauvert et al. 2019) mock communities. The pipeline used for the identification of bacterial and cyanobacterial ASVs in the Eco-AlpsWater project was based on DADA2 v. 1.16.0, under R (Callahan et al. 2016, 2018). The pipeline has been described,

with test samples, in Salmaso et al. (2021a).

The DADA2 bioinformatic pipeline for the analysis of the 18S rRNA HTS reads targeting the protists has been also adapted from the protocols proposed by Callahan et al. (2016, 2018) (https://benjjneb.github.io/dada2/index.html). Specifically, the bioinformatic pipeline used in the Eco-AlpsWater Consortium has been described (with test samples) in Salmaso et al. (2021b). Especially in protists, high caution is required in the evaluation and interpretation of ASVs diversity, due to the different 18S rRNA gene copies in the microeukaryotic cells (from less than a hundred, to well over half a million in ciliates), which can affect intragenomic heterogeneity and ASVs diversity (Wang et al. 2017; Salmaso et al. 2020).

On of the two bioinformatic pipelines chosen initially for the analysis of *rbcL* marker for diatom sequences cleaning and species identification used the "Mothur" software (Schloss et al. 2009). This pipeline allows processing of DNA reads produced by High-Throughput Sequencing technologies (Illumina MiSeq), from raw data to final OTU/Taxonomic inventories. This pipeline has been adapted to diatom DNA metabarcoding and already applied in different studies targeting benthic diatom communities in lakes (e.g. Rimet et al. 2018; Rivera et al. 2018) and rivers (Vasselon et al. 2017a b; Keck et al. 2018a, b). Descriptions of the different commands and the parameters used are also found in the Mothur wiki (https://www.mothur.org/wiki/). More recently, a second pipeline for the analyses of HTS diatom data used in the Eco-AlpsWater Consortium was based on the use of DADA2 protocols and identification of ASVs (see e.g. Tapolczai et al., 2019, and references therein; and https://github.com/fkeck/DADA2_diatoms_pipeline).

Bio-informatics processing of the eDNA metabarcoding data obtained with the MiFish-U primers (Miya et al. 2015) targeting the 12S fish marker gene was performed using the OBITOOLS3 software (Boyer et al. 2016). The protocol used allows the identification of ASVs, the final output of the analyses is a tab-delimited table with taxonomic inventories. A workflow example is available online (see https://git.metabarcoding.org/obitools/obitools3/wikis/Wolf-tutorial-with-the-OBITools3) and detailed descriptions of the individual commands can be found as well, https://git.metabarcoding.org/obitools/obitools3/wikis/The-OBITools3-syntax

❖ Integration of EAW procedures with current monitoring activities implemented in the Alpine Space region

Since its implementation, the European Water Framework Directive (WFD, 2000/60/EC) promoted freshwater biomonitoring for evaluating and improving aquatic ecosystem status through focused management plans. A range of organisms are used worldwide as indicators (Biological Quality Elements, BQEs) to monitor the quality status of aquatic ecosystems. These BQEs include phytoplankton, phytobenthos, aquatic plants, macroinvertebrates, and fish (EEA 2012, Fig. 4). Metrics and indices computed by using these indicators are used to obtain an Ecological Quality Ratio, which provide a direct measure of the ecosystem status.

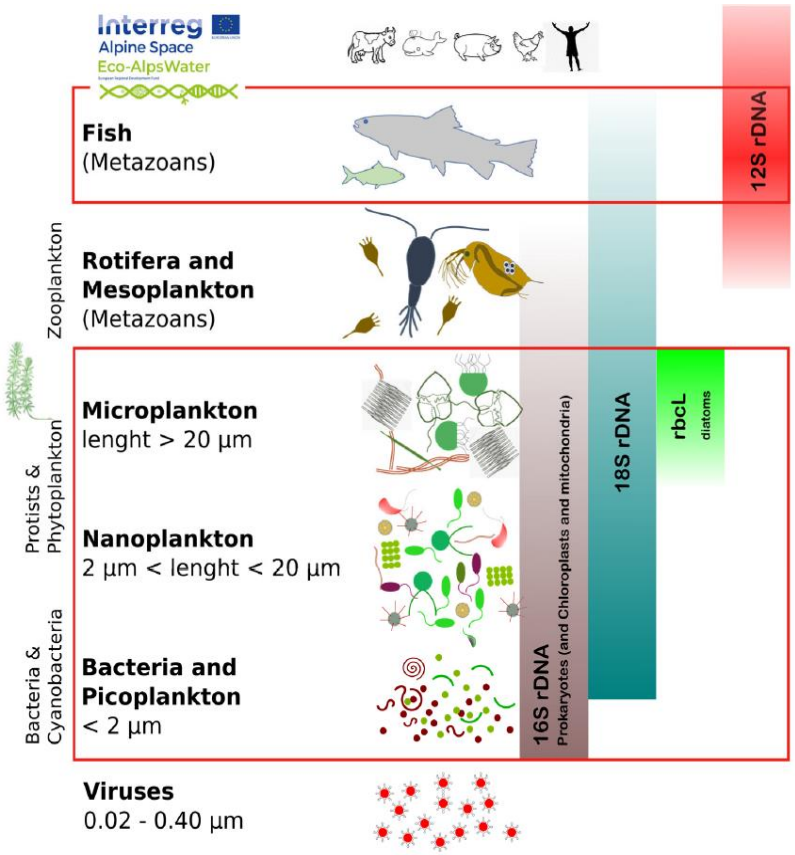

Figure 4. Biological Quality Elements considered within the EAW project.

In freshwater environments, most of the investigations were historically addressed towards the study of microalgae, which include both pelagic organisms (phytoplankton and cyanobacteria; Reynolds, 2006) and organisms attached to substrata, such as diatoms (Rimet et al. 2015) and other periphytic algae, either eukaryotic (Wehr and Sheath, 2003) or prokaryotic (Quiblier et al. 2013).

Besides cyanobacteria, phytoplankton is one of the main biological elements included in the Water Framework Directive for the evaluation of lake water quality (Water Framework Directive, 2000; Pasztaleniec, 2016). The use of phytoplankton and cyanobacteria in the assessment of water quality has been fostered by a long tradition of investigations based on the identification of species by light microscopy (LM) and polyphasic approaches, supplementing LM with genetic methods (Kurmayer et al. 2015; Shams et al. 2015; Wilmotte et al. 2017). Genetic analyses for taxonomic identification of organisms were traditionally performed on isolated strains using a range of rRNA markers (16S and 18S, and many other more selective genetic markers); for example, in the case of the taxonomic characterization of diatoms, *rbcL* markers have been widely used (Vasselon et al. 2017a, b; Rimet et al. 2018). Nevertheless, in the conventional biomonitoring programs, the identification of organisms is exclusively carried out by using morphological characters both under the microscope (microalgae) and by visual inspection of organisms (e.g., fish). The use of the metabarcoding approaches in the Eco-AlpsWater campaigns allowed to include systematically the molecular methods into the conventional biomonitoring plans, avoiding the limitations imposed by the isolation and cultivation of strains.

The sampling methods officially recommended for the biodiversity assessment of river and lake fish by the Water Framework Directive are electrofishing and gill netting, which are quite time consuming and therefore expensive methods and in addition require a numerous and qualified staff to be performed. Furthermore, electrofishing is restricted to the shallow littoral shoreline (CEN, 2003), to wadable rivers and to high-conductivity waters. Several sampling practices (e.g., seines or trawling) can severely damage the habitat and represent a danger, especially to rare species, and can heavily affect fish abundance in large lakes. Moreover, the classical freshwater biomonitoring practices require good taxonomic expertise for the morphological identification of the specimens and these methods cannot easily detect low abundant or elusive

species (the probability of detecting species that represents < 1% of the total abundance is exceptionally low, Paller 1995), thus providing partial estimates for entire communities (Deiner et al. 2017). While traditional fish sampling shows several shortcomings regarding biodiversity estimates, water sampling for eDNA analyses of fish proved to be potentially cheaper, easier, more sensitive, non invasive and more suited for surveys on extremely difficult sites. Reduced operational costs could allow regular sampling throughout the year, providing time series data and enabling systematic monitoring of fish biodiversity (and in general of community diversity) in different seasons and during extreme events (extremely dry seasons or floods). Conversely, traditional sampling can usually be performed once or twice a year.

eDNA metabarcoding is revealing one of the tools of choice of the 21st century for fundamental research and the future of large-scale biodiversity monitoring programs, thanks to its cost-effectiveness and easy implementation. The biodiversity assessment of microorganisms using eDNA metabarcoding has overcome the limitations of light microscopy identification of species, providing a more refined description of community composition, and allowing the identification of potentially dangerous, rare, and previously undetected taxa (Keskin et al., 2016; Marshall et al., 2019). For fish biodiversity surveys the method is still in its infancy but it is already showing its effectiveness in describing fish community composition (Pont et al. 2018) and the species richness computed on eDNA fish metabarcoding data was found comparable to the cumulative number of species collected during long-term electrofishing and gill netting surveys (e.g. Pont et al. 2018, Hänfling et al. 2016, Riccioni et al., submitted). Moreover, the ability to identify DNA present in traces represent an important advantage for the early detection of exotic and potentially invasive species and the tracking of elusive endangered species (Pawlowski et al. 2018).

The combination of traditional monitoring approaches and up-to-date tools, based on eDNA metabarcoding analyses, grants a more comprehensive description of water ecological status representing an unmatched step forward for an improvement of management plans of Alpine water resources. A thorough description of aquatic biological communities provides a fundamental tool for the protection and valorisation of the ecosystem services represented by lake and river ecosystems. These ecosystem services include provision services (including

drinking water supply and recreation), primary productivity supporting services, biodiversity supporting services, water purification regulating services. However, no metrics, as used for the status assessment based on traditional methods, have been developed for eDNA yet. These metrics need to be defined in the order to further improve the analysis and significance of data obtained by molecular monitoring methods.

A better evaluation of ecosystem status also allows a better evaluation of risk management and is a further step towards rational choice and policy implementation with important consequences for the safeguarding of the whole Alpine space. The creation of a well connected, long-term network throughout the Alpine region promoted by the EAW project provides the groundwork to a homogeneous management and protection of ecosystem resources. The guidelines provided represent a crucial tool to evaluate ecosystem health, promoting the application of focused policies for the sustainable use of ecosystems.

Annex 1

Within the WP1, and with the support of WP2 outputs, formalised methods for each biomonitoring activity have been produced after careful on-site evaluation. Below, the list of the individual protocols for the the collection and analysis of eDNA is reported (steps summearized in Fig. 3). These original, technical protocols include the long, full developmental stages of all the field and laboratory methods tuned and adapted within the EAW project. A selection of these protocols has been further revised (e.g. fo publication in protocols.io and zenodo) and included in the web pages of the project: https://www.alpine-space.org/projects/eco-alpswater/en/project-results/all-documents, section **'WP1 - "protocols"'**.

A synthetic and systematic description of the final protocols have been included in the e-booklet 2 https://www.alpine-space.org/projects/eco-alpswater/publications-booklets/2-en--technical-guidelines.pdf

- **Sampling procedure guidelines**

Different protocols have been set-up to adapt the sampling procedures to the different matrices to collect (biofilm, water) and to the different target organisms.

- Deliverable D.T1.1.2.-1 is a guideline for collecting lake plankton samples. The sampling strategy is like that used for classical phytoplankton survey focusing on the euphotic zone, however the procedure for filtration and preservation is adapted for DNA samples. It provides a reliable and replicable method for the sampling of lake micro-plankton to be used for downstream DNA analysis.
- Deliverable D.T1.1.2.-2 and 3 describes the recommended procedures for lake and river biofilm sampling. This field protocol is optimised for routine sampling and agrees with CEN guidance (NF EN 13946) and CEN technical report (FprCEN/TR 17245) for the analysis of benthic diatoms from rivers and lakes. The application proposed here in the context of Eco-AlpsWater

aims at comparing DNA inventories to traditional inventories (microscopy).

- Deliverable D.T1.1.2.-3 describes eDNA fish sampling collection. 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. The sampling design varies between lakes and rivers.

- **DNA extraction procedure guidelines**

Different DNA extraction protocols have been set-up for the different type of material/filter used (biofilm/Sterivex/filters or VigiDNA cartridges) and to the different target organisms; other protocols have been tested (see text and references).

- Deliverable D.T1.1.2.-6 Plankton DNA extraction. This protocol is part of the DNA workflow applied in the Eco-AlpsWater Project, here in particular to characterize the diversity of plankton in lakes. The protocol described in the deliverable uses the DNeasy® PowerWater Sterivex Kit (QIAGEN) with specific modifications adapted to plankton DNA extraction.
- Deliverable D.T1.1.2.-7 Biofilm DNA extraction. The choice of the methodology for biofilms DNA extraction is based on previous studies and on the work done by Vasselon et al. (2017). The DNA extraction protocol presented below is based on a protocol adapted from the NucleoSpin® Soil kit (MACHEREY-NAGEL) with specific modifications for biofilm DNA extraction.
- Deliverables D.T1.1.2.-8.1 and 8.2 Fish DNA extraction. The choice of the methodology for fish DNA extraction is based on previous studies with some adaptations for the two different filtering systems used (Sterivex and VigiDNA cartridges). The DNA extraction protocol presented below is based on a protocol adapted from the NucleoSpin®Soil kit (MACHEREY-NAGEL) with specific modifications according to the detailed protocol presented by (Pont et al. 2018) and the tests done in the context of the Eco-AlpsWater project.

- **HTS DNA library preparation guidelines**

  - Deliverable D.T1.1.2.-9 This deliverable describes all the steps in the DNA workflow, i.e., PCR amplification of selected barcodes, and wet lab methods to prepare DNA library for downstream MiSeq Sequencing to be used for rbcL marker gene analyses for diatoms metabarcoding assays.
  - Deliverable D.T1.1.2.-10 Illumina library preparation protocol for bacterioplankton communities. This deliverable provides a synthetic description of the Illumina library preparation protocol for eDNA metabarcoding analyses of bacterial communities based on 16S marker.
  - Deliverable D.T1.1.2.-11 Illumina library preparation protocol for eukaryotic microplankton communities. This deliverable provides a synthetic description of the Illumina library preparation protocol for eDNA metabarcoding analyses of eukaryotic communities based on 18S marker.
  - Deliverable D.T1.1.2.-12 Illumina library preparation protocol for freshwater fish communities. This document provides a detailed description of the Illumina library preparation protocol for eDNA metabarcoding analyses of freshwater fish communities based on 12S marker, previously assessed and verified through an intercalibration test (Deliverable D.T1.1.2).

- **Bioinformatic analyses guidelines**

These guidelines describe in detail the main steps of the bioinformatic process applied to treat high throughput sequencing (HTS) data for metabarcoding analysis produced within the Eco-AlpsWater project.

These protocols are those proposed by the Eco-AlpsWater consortium to promote the implementation of HTS of environmental DNA in the biomonitoring and ecological assessment of water bodies (lakes and rivers).

These guidelines are the result of an accurate evaluation of the bioinformatic protocols currently

used by the scientific community for the analysis of metabarcoding data: each marker gene is therefore analysed by using different pipelines that showed the best performance in previous studies.

- Deliverable D.T1.1.3-1 Diatom DNA metabarcoding bioinformatics pipeline. This protocol describes in detail the main steps of the bioinformatics process applied to treat high throughput sequencing (HTS) data, in particular for Diatoms metabarcoding. The pipeline suggested for the rbcL marker gene is Mothur. This protocol was used in different studies targeting benthic diatom communities from lakes (e.g., Rimet et al., 2018; Rivera et al., 2018) and rivers (Vasselon et al., 2017a, b; Keck et al., 2018a, b).
- Deliverable D.T1.1.3-2A Metabarcoding protocol – Analysis of Bacteria (including Cyanobacteria). The pipeline suggested for the 16S marker gene (Bacteria including Cyanobacteria) is Dada2. This deliverable reports a pipeline, based on DADA2 v. 1.16.0, under R, for the identification of ASVs from 16S HTS data. The pipeline has been adapted from those continuously updated from the WEB site of DADA2 (https://benjjneb.github.io/dada2/index.html, Callahan et al., 2016, 2018).
- Deliverable D.T1.1.3-2B Metabarcoding protocol – Bacteria and cyanobacteria 16S rRNA gene. This is an alternative procedure to the Dada2 analysis protocol. Edmund Mach Foundation (LP of Eco-AlpsWater project) has developed a set of bioinformatic tools for the filtering, quality control, and processing of metagenomic and metabarcoding reads that are integrated into an actively updated and maintained pipeline (MICrobial Community Analysis - MICCA; Albanese et al., 2015).
- Deliverable D.T1.1.3-3 Metabarcoding protocol – Protists, including Phytoplankton. This deliverable reports a pipeline, based on DADA2, under R, for the identification of ASVs from18S marker gene HTS data (Protists including Phytoplankton). The pipeline has been adapted from those continuously updated from the WEB site of DADA2 (https://benjjneb.github.io/dada2/index.html, Callahan et al., 2016, 2018).
- Deliverable D.T1.1.3-4 Bio-informatic pipeline for the analyses of eDNA metabarcoding data for fish communities. This protocol provides a detailed description of the main steps of the bio-

informatics pipeline for the processing of HTS data for fish metabarcoding analysis. This protocol focusses on the bio-informatic processing of the eDNA metabarcoding data obtained with the MiFish-U primers (Miya et al., 2015, 12S marker gene) using the OBITOOLS3 software (Boyer et al., 2016).

- Deliverable D.T1.1.4 Ecological metrics produced from diatom DNA metabarcoding HTS data. Using the normalized diatom taxonomic list, several water quality indices based on diatom genus and species lists can be computed to evaluate the ecological status of water (for each environmental sample). This step is performed using the OMNIDIA software which includes ecological preferences related to each taxon and different water quality indices (e.g., IPS, IBD).

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