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Perspectives on eDNA monitoring in Alpine waters

2021

FOR STAKEHOLDERS AND DECISION-MAKERS

Eco-AlpsWater

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

Exciting results, perspectives, guidelines and policy recommendations for eDNA monitoring and more ...

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Contents

All actions in the environment are reflected in the health of our waters. Eco-AlpsWater have optimised the metabarcoding tool for more effective management and protection of ecosystem services in Alpine waters.

Premise

The aim of this booklet is to provide a general overview of the results achieved by the Eco-AlpsWater project. Furthermore, the results will be interpreted highlighting the potential that high-throughput sequencing techniques will have in upgrading conventional water biomonitoring approaches. Part of the information provided in this booklet has been taken from the deliverables published on the Eco-AlpsWater (EAW) web pages dedicated to the Project results. These documents include full information and a complete list of supporting references.

Results overview and perspectives

One of the main objectives of the project Eco-AlpsWater (EAW) was to develop and implement innovative high throughput DNA sequencing methods (HTS) to complement and improve the EU Water Framework Directive and, in Switzerland, the Water Protection Ordinance standards. The biological elements included in the investigations were cyanobacteria, other eukaryotic microalgae, and fish (Fig. 1). The general strategy adopted by the project was common to all these biological elements, i.e. it was based on a simultaneous collection of samples for HTS (Table 1) and traditional (Table 2) laboratory analyses (Fig. 2).

Table 1. Biological elements analysed using high throughput sequencing. The protocols used to analyse the gene markers have been reported in the Brochure #2.

Target Biological elements	**Gene markers**	**Other target taxonomic groups detected**	**Other non-target taxonomic groups detected**	**Habitats**
Cyanobacteria	16S rRNA	Heterotrophic bacteria	Chloroplasts, mitochondria, Archaea (rare)	PL lakes; BFM of lakes and rivers
Eukaryotic microalgae (including diatoms)	18S rRNA	Heterotrophic protists; fungi	Selected metazoans (e.g. zooplankton); ciliates; higher plants (macrophytes)	PL lakes; BFM of lakes and rivers
Diatoms	rbcL	-	-	BFM lakes and rivers
Fish	12S rRNA	-	Other metazoans (including mammals)	Lakes and rivers

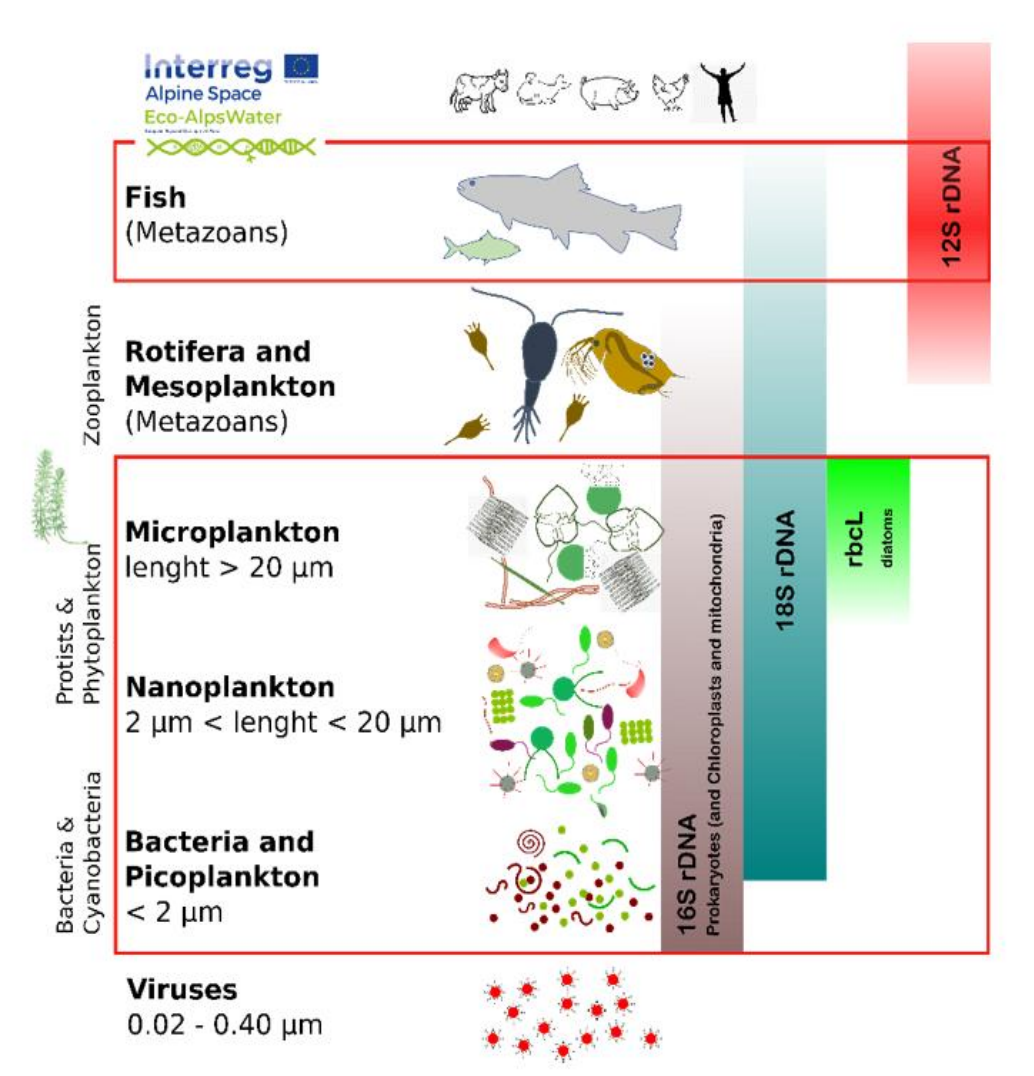


Fig. 1 – Schematic representation of planktonic organisms and nekton in freshwater bodies. The biological elements included in the monitoring activities of the project EAW are enclosed within red squares; these include bacteria and cyanobacteria, protists (including photosynthetic and mixotrophic microalgae, and pelagic and benthic diatoms), and fish. Macrobenthos is not represented. The specific genes used in the project are intended to target bacteria/cyanobacteria (16S rRNA gene), unicellular protists (18S rRNA gene), diatoms (rbcL), and fish (12S rRNA gene). Nevertheless, though designed to amplify genetic regions belonging to these intended organisms, the generality of primers is such as to amplify also "unintended" biological elements, such as, e.g., chloroplasts and mitochondria (16S rRNA gene), metazoans (mostly zooplankton, 18S rRNA gene), and higher organisms (such as mammals, 12S rRNA gene). Size classes are indicative; microorganisms (as well as other organisms) are not in scale (from: https://doi.org/10.5281/zenodo.5233527).

Table 2. Biological elements analysed using traditional approaches (species determination using morphological criteria)

Target Biological element	Method	Sample treatment	Other non-target taxonomic groups detected	Habitat
Cyanobacteria	Light Microscopy	Lugol's fixation and Utermöhl counting	-	PL lakes; BFM of lakes and rivers (AT)
Eukaryotic microalgae (including diatoms)	Light Microscopy	Lugol's fixation and Utermöhl counting	-	PL lakes; BFM of lakes and rivers (AT, SI)
Diatoms	Light Microscopy	Oxidation and cleaning of siliceous frustules	-	BFM lakes and rivers
Fish	Direct observation	Electrofishing / nets	-	Lakes and rivers (selected habitats)

In addition to the plankton (PL) and nekton habitats in lakes, analyses were also addressed to the biofilm (BFM) collected on stones in lake and river shores. Traditionally, in most countries these habitats are investigated by studying exclusively the diatom communities (see brochure 2) (Tables 1, 2 and Fig. 2).

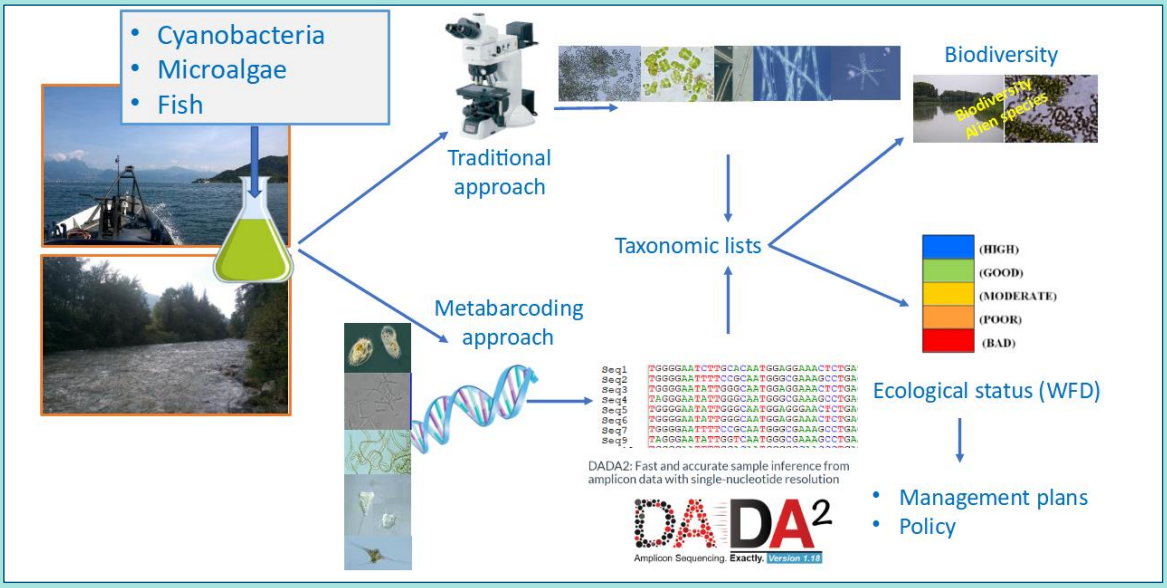


Fig. 2. EAW main approach. Water and biofilm samples, collected in lakes and rivers, were analysed using traditional (morphological taxonomic determination of specimens) and metabarcoding (DNA-based analyses) approaches. The taxonomic lists of species obtained by the two methods are integrated, allowing to obtain a more robust assessment of biodiversity. In turn, the list of species is used at different levels of analysis, i.e. for the computation of biological indices and the identification of new organisms, including non indigenous species.

As for the HTS analyses, only the "Target Biological elements'' are considered in the WFD ecological status assessment (Table 1). The remaining groups ("Other target taxonomic groups detected") are purposely covered by the primers used for the library preparation, but they are not considered by the present WFD bio-assessment criteria. The column "Other non-target taxonomic groups detected" lists a selection of groups that, though not specifically targeted by the selected primers, can still be amplified. In some cases, the information gathered for these groups can provide ancillary information to be used for taxonomic classification (e.g. 16S rRNA genes in

Chloroplasts). In the eukaryotes (18S rRNA genes), some "non-target" taxa may be detected with a high number of reads, as the large zooplankters (e.g. copepods) or, with a smaller number of reads, giving important indications about the presence of large non indigenous species (NIS) metazoans, such as *Dreissena rostriformis* (*D. bugensis*; quagga mussel) in lakes Leman and Bourget.

The collection and analysis of biological samples was complemented by a concurrent series of measurements of environmental variables on the field and by a collection of samples to be subjected to chemical and physical analysis in the laboratory (Table 3). Finally, a selection of samples was analysed to assess the concentrations of a wide spectrum of cyanotoxins analysed by liquid chromatography / mass spectrometry (Table 4).

Table 3. Environmental variables analysed in the field and laboratory. Major ions were determined only on a selection of water bodies.

Environmental data	**Method**	**Unit**
Temperature	Field measurement	°C
pH	Field measurement and laboratory	
Conductivity	Field measurement and laboratory	µS cm^{-1} 25°C
Light attenuation coefficient	Field measurement	m^{-1}
Secchi disk depth	Field measurement	m
Euphotic layer	Field measurement	m
Oxygen	Laboratory	mg L^{-1}
Oxygen (%)	Laboratory	%
Total alkalinity	Laboratory	mg L^{-1}
Bicarbonates	Laboratory	mg L^{-1}
Nitrate nitrogen	Laboratory	µg N L^{-1}
Sulphates	Laboratory	mg L^{-1}
Chloride	Laboratory	mg L^{-1}
Calcium	Laboratory	mg L^{-1}
Magnesium	Laboratory	mg L^{-1}
Sodium	Laboratory	mg L^{-1}
Potassium	Laboratory	mg L^{-1}
Ammonium	Laboratory	µg N L^{-1}
Total nitrogen	Laboratory	µg N L^{-1}
Soluble reactive phosphorus	Laboratory	µg P L^{-1}
Total phosphorus	Laboratory	µg P L^{-1}
Reactive silica	Laboratory	mg Si L^{-1}
Dry weight	Laboratory	mg L^{-1}
Chlorophyll a	Laboratory	µg L^{-1}

Table 4. Cyanotoxins analysed by means of liquid chromatography and mass spectrometry (LC-MS) in the water and biofilm samples. The analyses were performed on a selection of water bodies. All the cyanotoxins congeners were measured at the level of ng L^{-1}.

Cyanotoxins	
Microcystins and nodularin	*(continuation of left column)*
MC-RR	*Anatoxins and Homoanatoxins*
MC-RRdm	ATX-a
MC-YR	Hatx-A
MC-YRdm	
MC-LR	*Cylindrospermopsins*
MC-LRdm	Cyn
MC-LA	DehydroCyn
MC-LAdm	
MC-LY	*Saxitoxins*
MC-LYdm	STX
MC-LW	NeoSTX
MC-LWdm	DecarbamoylSTX
MC-LF	GTX1
MC-LFdm	GTX2
MC-HtyR	GTX3
MC-HtyRdm	GTX4
MC-WR	GTX5
MC-WRdm	C1
Nodularin-R	C2

The collection of samples and analyses were carried out on 37 lakes and 23 rivers (Fig. 3). Of these, a number of key lakes and rivers were analysed with higher temporal frequency (ca. monthly) or greater spatial coverage. These water bodies are highlighted in red in the two maps of Fig. 3.

The water bodies analysed are characterized by large differences in the morphometric and morphological characteristics. The range of surface, maximum depth, and altitude of the lakes included in the research are between 0.0066 and 582 km^2, 1.3 and 410 m, and ca. 1 m and 2125 m above sea level. Similarly, the length of rivers span from a very few km to several hundreds of km typical of the large European rivers (e.g. River Sava, 945 km).

The approach adopted in the EAW project was based on a strict cooperation between 12 partners, which allowed to collect over 300 samples for the HTS analyses of cyanobacteria and eukaryotic microalgae/diatoms in the open water of lakes (pelagic samples) and along the shores of lakes and rivers (biofilm samples), and over 220 samples for the HTS analysis of fish. While all microorganism samples analysed with HTS were followed by a corresponding microscopic analysis, for fish most of the analyses were performed using HTS approaches only. In this latter case, the comparison between the two methods was performed by using the conventional electrofishing or net samplings of fish performed in previous periods/years or soon after the eDNA sampling campaign (e.g., within one hour in Lake Starnberg and River Wertach).

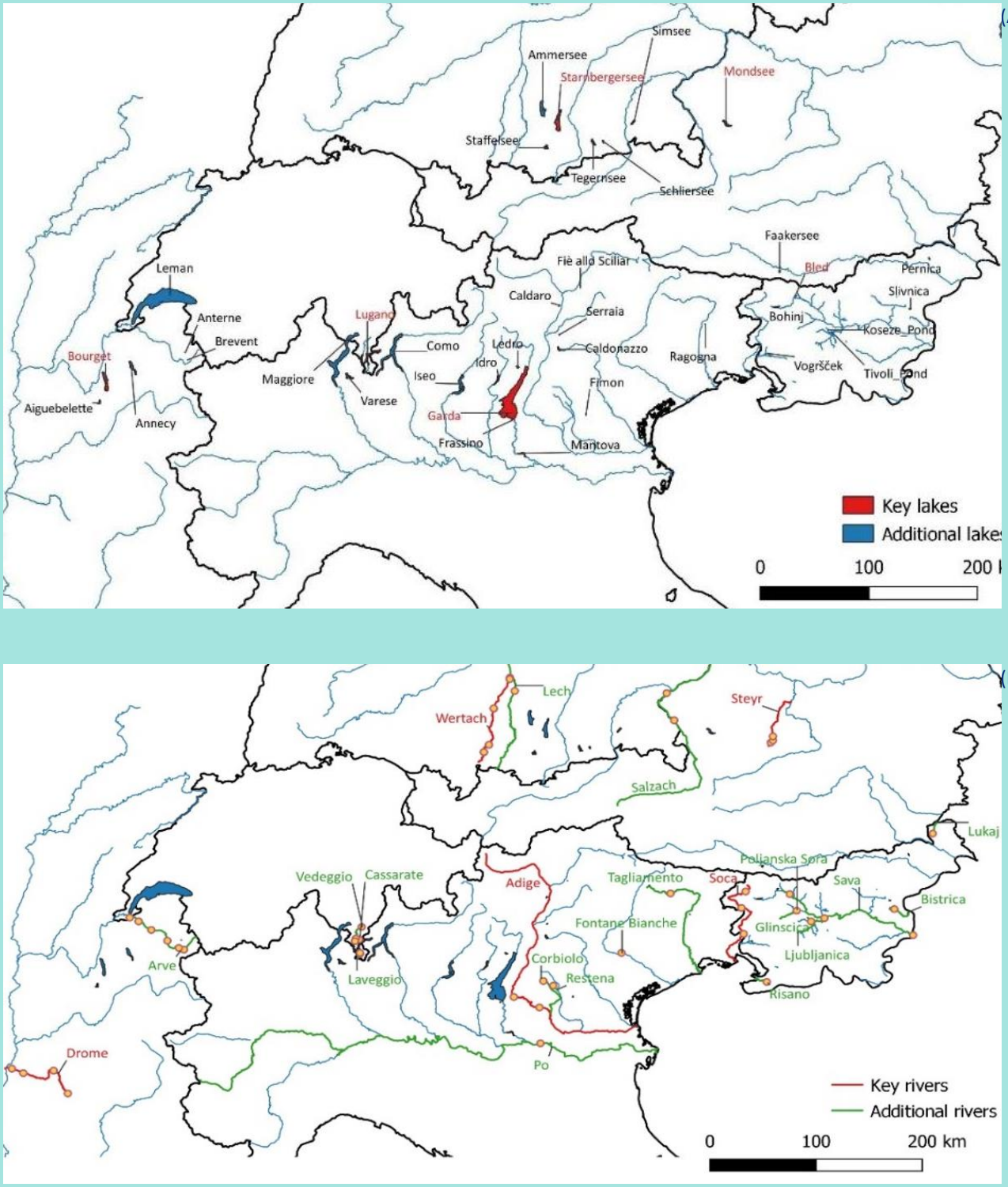


Fig. 3. Location of (A) 37 lakes and (B) 23 rivers investigated during the study. The key water bodies highlighted in red have been sampled more frequently (A) or with greater spatial coverage (B) than the additional water bodies (green).

By limiting the attention only to the biological elements analysed by HTS and the 16S and 18S rRNA marker genes, a few numbers can provide an idea of the order of magnitude of the amount of data collected during the investigations (Table 5).

Table 5. HTS analysis of microorganisms in the EAW project. The bioinformatic analyses of the FASTQ samples were performed adapting the DADA2 pipeline and using the reference taxonomic databases SILVA v. 138 and PR2 v. 4.14.0. The number of families and species is based on the taxonomic annotation by using the Naïve Bayesian Classifier implemented in DADA2, and a conservative 95% bootstrap level (detailed protocols for the 16S rRNA and 18S rRNA, as well as rbcL and 12S rRNA genes are reported in Brochure # 2).

	16S rRNA	**18S rRNA**
Water bodies	60	60
Sampling stations	260	260
Samples collected and extracted	330	330
Number of total reads (F&R) in FASTQ files	42,000,000	53,000,000
Final raw sequences (not rarefied) after quality filtering, merging and chimera removal (Amplicon Sequence Variants, ASVs)	52,455	21,371
Raw sequences (not rarefied) after taxonomic filtering and removal of undetermined phyla/divisions	46,330	13,454
Bacteria - Number of families	324	-
Bacteria - Number of genera	832	-
Cyanobacteria - Number of families	27	-
Cyanobacteria - Number of genera	72	-
Protists and fungi - Number of families	-	331
Protists and fungi - Number of genera	-	655
Eukaryotic microalgae - Number of families	-	83
Eukaryotic microalgae - Number of genera	-	236

The high number of genotypes (ASVs) found after the application of the bioinformatic pipelines to the raw reads (over 50,000 bacteria and over 20,000 protists and fungi) is only partly due to the large number of samples analysed. Actually, most of the diversity originates from the high diversity in the physiographic characteristics, geographical location of the water bodies and microhabitat analysed (Fig. 4).

In the framework of the project, the HTS data are mainly used to integrate the taxonomic data recorded by using the traditional morphological criteria and traditional biological elements included in the WFD/WPO. A first selection of the results obtained in lakes and rivers is reported on the project website (section Project results, WP3 - "pilots", Report on 6 key lakes and Report on 6 key rivers).

At present, one of the major limitations in the application of HTS approaches to aquatic biomonitoring is the incompleteness of reference databases and the short length of the DNA reads generated by the most commonly used sequencing technologies. In perspectives, these shortcomings will have to be addressed by: i) expanding the use of primers that allow for higher specificity in conjunction with dedicated databases (such as the Diat.barcode, used to classify diatoms); ii) the adoption of advanced HTS technologies providing longer DNA reads for metabarcoding assessment; iii) the use of environmental genomic approaches.

Compared with the fraction of HTS data that are utilized in the comparison with the traditional biological elements included in the WFD/WPO, the extent of unexploited information is very high (e.g. Table 5). Historically, biological elements have been selected among those suitable for taxonomic classification based on morphological attributes and for which there was a consolidated tradition of observations, taxonomic manuals, and investigations on ecological and economic implications and impacts (e.g. phytoplankton/cyanobacteria,

fish). With the advent of successive generations of "Next Generation Sequencing" approaches, the obstacles that cause the exclusion of heterotrophic bacteria, protists and heterotrophic fungi, and other biological elements not considered in this research (Fig. 1) are no longer justified. However, although practical applications in this direction are still very scarce (EAW website, DT1.4), the assessment of ecological integrity and ecosystem services based on the use of a wider range of biological elements is one of the most promising research fields.

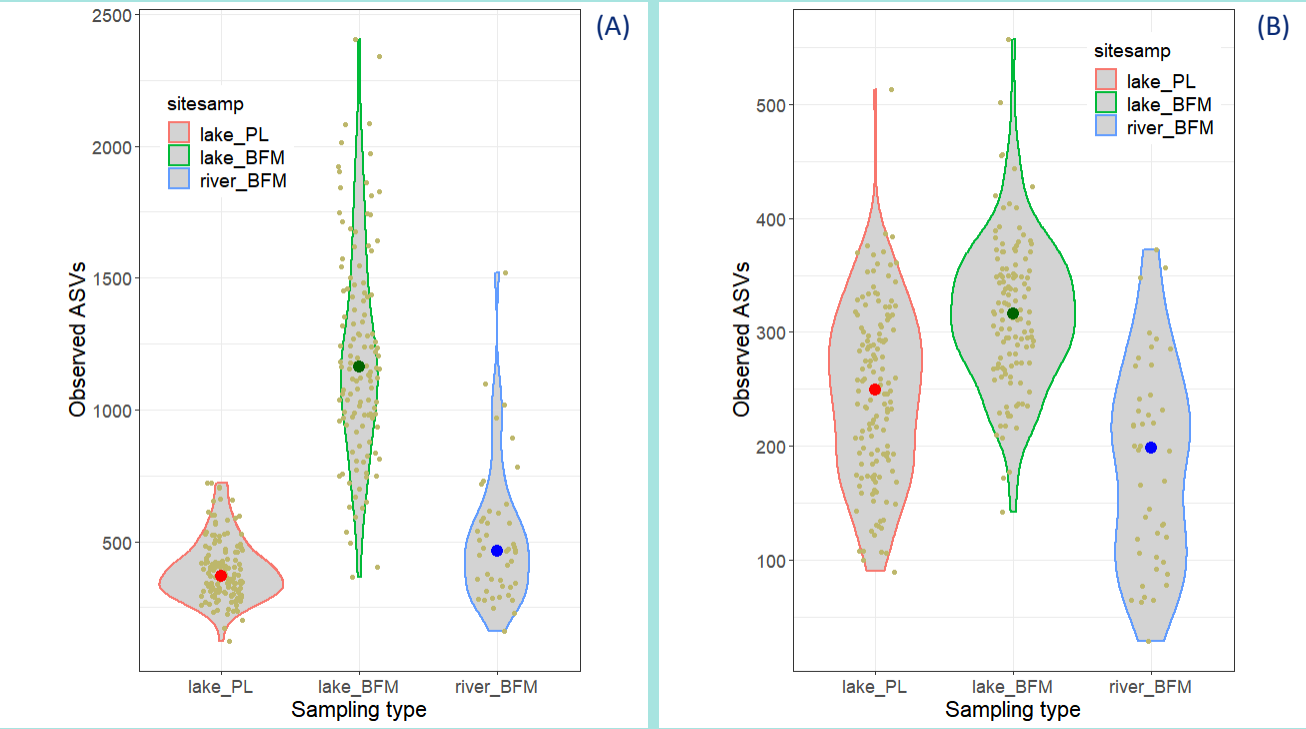


Fig. 4. Number of amplicon sequence variants (ASVs) obtained after taxonomic filtering and removal of undetermined phyla/divisions in the three main sample typologies, cf. Table 5. The violin plots show the probability density of the data, smoothed by a kernel density estimator on each side. PL, plankton samples in lakes; BFM, biofilm samples in lakes and rivers.

Results from key lakes and rivers

(from deliverables D.T3.2.1, D.T3.2.2, D.T3.2.3 and D.T3.3.1)

All 6 key lakes (Bled, Bourget, Garda, Lugano, Mondsee, Starnberger See) are included in the L-AL3 lake type of the Alpine GIG (lowland or mid-altitude, deep, moderate to high alkalinity with alpine influence, large). During different limnological seasons, the temperature of water at sampling campaigns was ranging from 3 to 27°C, with conductivity from 209 to 355 µS/cm. In our key lakes we have gathered 157 lake samples composed of 78 plankton samples.

For **phytoplankton** the euphotic layer was sampled in a depth-integrated manner. The trophic status of key lakes has been assessed by three parameters: total phosphorus, transparency and chlorophyll-*a* concentration and analysed with OECD fixed boundary trophic classification system (OECD; 1982). According to total phosphorus concentrations the majority of samples from key lakes are classified oligotrophic (68%), followed by mesotrophic (32%). Similarly, according to transparency (Secchi depth) the majority of samples are classified as oligotrophic (49%), followed by mesotrophic conditions (42%). On the other hand, according to chlorophyll-*a* concentrations the majority of samples from key lakes are classified mesotrophic (60%), followed by oligotrophic state (35%). In summary the vast majority of lake plankton samples are assigned to the oligo-mesotrophic state (Fig. 5).

Overall (very) good qualitative relationship between sequencing results (HTS reads) and microscopy (biovolume) was obtained. Thus sequencing results can be used to confirm microscopical counting based on morphological characters, i.e. the differentiation of the genus *Tychonema* from *Planktothrix* based on subtle morphological characters. In general the mismatch in positive detection between microscopy biovolume vs sequencing was rather low (0-10%), i.e. very little “false positives” were obtained through microscopy. On the other hand, in general for all taxa the sequencing was found more sensitive than the biovolume estimate via microscopy (3 – 30 (50) % higher positive detection rate). Not surprisingly, in the microscope the biovolume of picocyanobacteria was underestimated and picocyanobacteria very likely were more reliably detected and quantified using HTS (reads). Nevertheless, two filamentous cyanobacteria with high identity to *Nodosilinea or Prochlorothrix* were detected by sequencing only, implying potential refinement of microscopical analysis. Overall also good quantitative relationships between HTS (reads) and (LM) biovolume were observed for many filamentous or colony-forming genera, i.e. even quantitative conclusions seem possible. Such a comparison is shown for a key phytoplankton species *Planktothrix rubescens* (Fig. 6).

Furthermore, it was interesting to see that populations of planktonic cyanobacteria were composed of variable numbers of partly co-occurring genotypes (oligotypes). The pic ocyanobacteria (*Cyanobium*) occurred with a maximum number of genotypes (i.e. 29), while *Tychonema* occurred with one genotype only. The rare genus *Prochlorothrix* occurred with six genotypes. Even for harmful and bloom-forming cyanobacteria such as *Microcystis* or *Planktothrix* characterized intensively using clonal isolates in the laboratory several new genotypes were found. Thus in contrast to microscopy and isolation of strains, by sequencing a more deep characterization of population genetic structure was achieved.

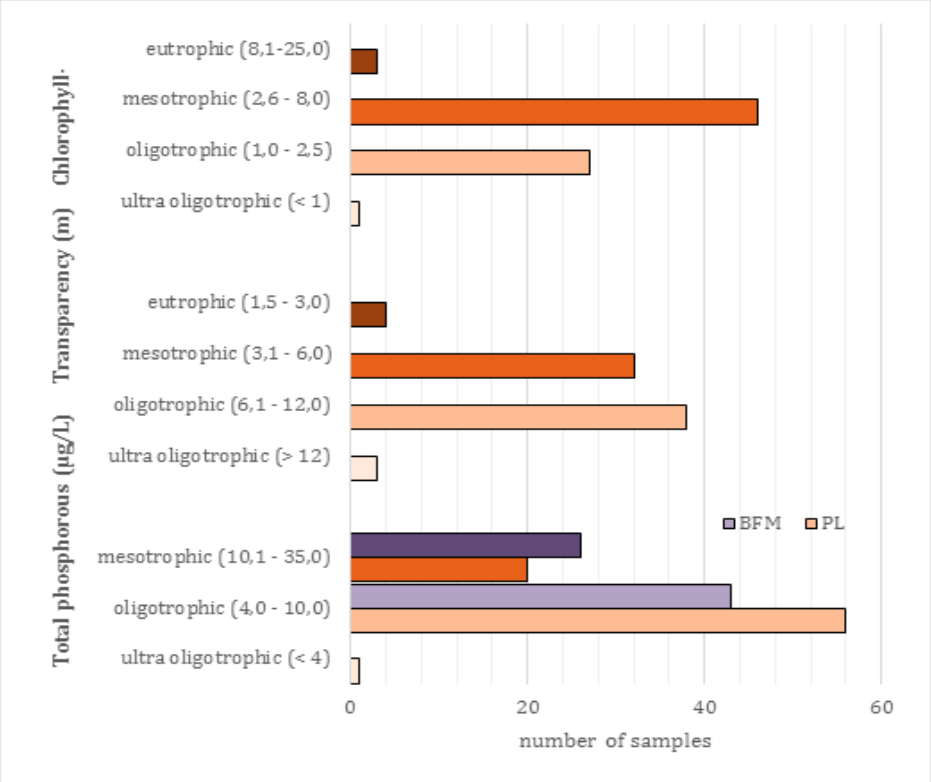


Fig. 5: Frequency distribution of samples from plankton (PL) according to trophic state derived from chlorophyll-a concentration (µg/L), transparency (Secchi depth) and total phosphorus concentration (µg/L), based on the trophic classification system (OECD; 1982).

Using 16S rDNA it is observed that 16S rDNA genotype composition informs about dominant taxa (e.g. the dominance of *D. lemmermannii* or *Aphanizomenon flos-aquae* under deep mesotrophic conditions). On the other hand 16S rDNA genotype composition can be used for surveillance and control of possibly invasive cyanobacterial taxa (e.g. members of Nostocales in consequence to climate warming), i.e. *Chrysosporum ovalisporum* and *Cylindrospermopsis raciborskii,* the latter identified for the first time in lakes Frassino and Mantova (NE Italy).

Using 18S rDNA the frequency of detection on genus level for all groups of eukaryotic phytoplankton was compared: Chlorophyta (green algae), Streptophyta (including Zygnemales and desmids), Bacillariophyta (diatoms), Cryptophyta (cryptomonads), Chrysophyta (chrysophytes as well xanthophytes and haptophytes) and Dinophyta (dinoflagellates). For many genera a good qualitative correspondence on genus level between both methods was observed, including flagellates, coccale, filamentous as well as colony-forming organization types. Overall a higher sensitivity for detecting eukaryotic microalgae (flagellates) was observed, also because of increased sampling volume (from 500 to 2000 ml of filtered volume vs 10-25 ml in sedimentation chambers). Thus additional information on certain groups of algae which have not been well recorded before, i.e. eukaryotic flagellates (Chrysophyceae, Dinophyta, Volvocales) but also entire new algal groups (Eustigmatophyta) has been obtained. For example chrysophytes, cryptomonads and dinoflagellates tend to occur with high biodiversity in lakes but are not differentiated in the microscope. On the other hand considerable information on interspecific variation among algal taxa not recognized by microscopy was obtained (i.e. Chrysophyceae, Volvocales, Bacillariophyceae).

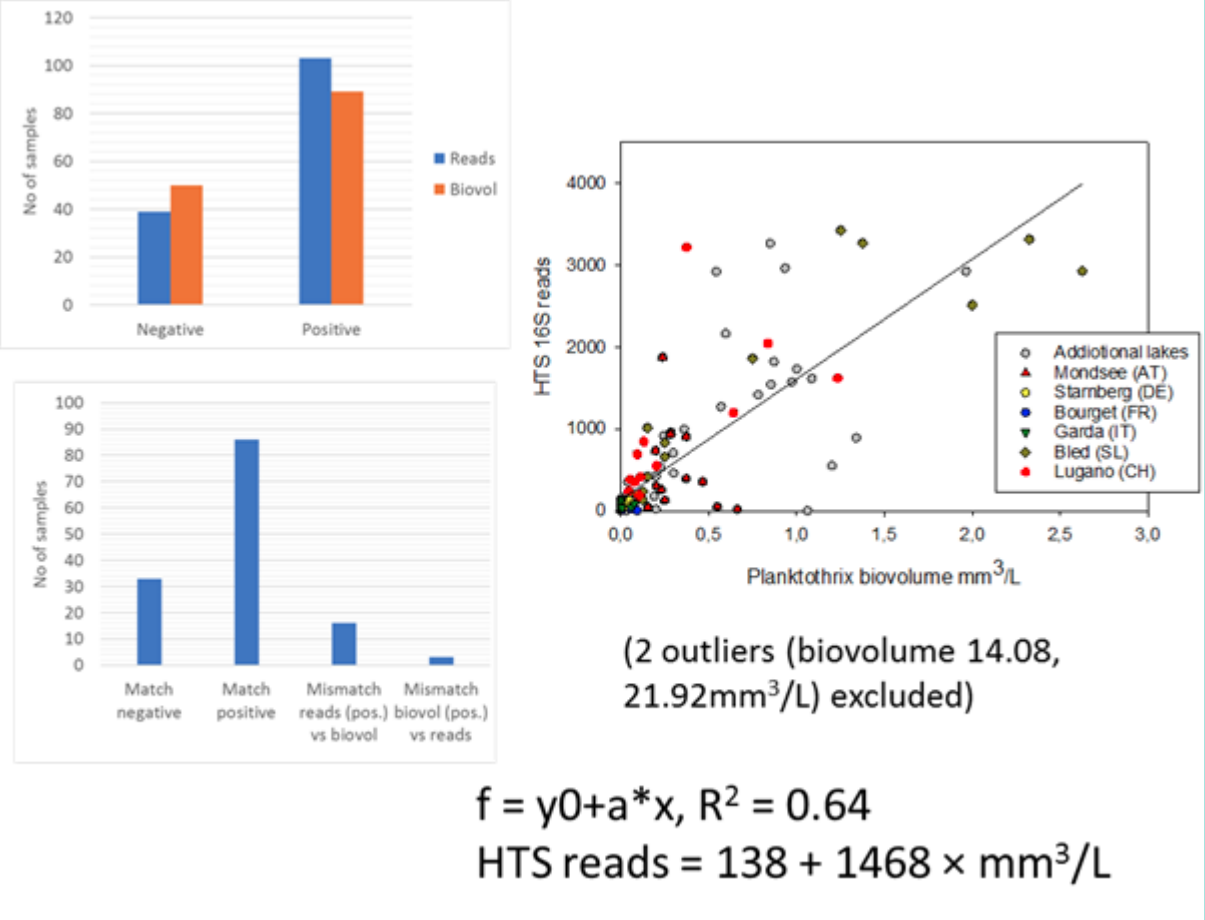


Fig. 6. Comparison of 16S rDNA sequencing vs microscopic counting (Planktothrix biovolume).

Eukaryotic Phytoplankton

Genus	HTS (18S)	LM (Biovol)
Botryococcus	37	7
Chlamydomonas	90	23
Coelastrum	12	11
Phacotus	63	33
Secenedesmus	53	51
Schroederia	3	3
Tetraselmis	29	14

Genus	HTS (18S)	LM(Biovol)
Closterium	48	45
Cosmarium	1	1
Mougeotia	10	8
Staurastrum	11	11

Bacillariophyta

Genus	HTS (18S)	LM(Biovol)
Asterionella	64	61
Aulacoseira	117	107
Cyclotella	28	28
Cymotopleura	2	1
Diatoma	10	3
Discostella	7	1
Fragilaria	106	88
Melosira	9	1
Navicula	9	5
Nitzschia	23	20
Stephanodiscus	126	111
Ulnaria	30	30
Urosolenia	9	3

Cryptophyta

Genus	HTS (18S)	LM(Biovol)
Cryptomonas	851	657
Plagioselmis	12	12
Rhodomonas	20	4

Chryso-Xantho-Haptophyta

Genus	HTS (18S)	LM(Biovol)
Epipyxis	23	3
Mallomonas	265	163
Ochromonas	26	9
Pseudopedinella	108	14
Synura	18	4
Uroglena	64	17
Tribonema	8	6
Chrysochromulina	201	81

Dinophyta

Genus	HTS (18S)	LM(Biovol)
Ceratium	161	124
Dinobryon	325	316
Dinophyceae	140	5
Gymnodinium	299	236
Peridinium	43	33

Fig. 7. Detection frequency for eukaryotic phytoplankton using both methods 18S rDNA sequencing and microscopic counting. Taxa have been assigned using REBECCA codes on genus level but typically include several species as identified under the microscope. The red marks indicate a significant lower frequency of detection using light microscopy.

The additional information regarding assessment of the **ecological status** via phytoplankton can be summarized as follows:

1) By HTS the occurrence of relevant taxa to calculate the Brettum index was confirmed, for example from Cyanobacteria (*Planktothrix, Aphanizomenon, Snowella*), diatoms (A*ulacoseira subarctica, Ulnaria ulna*), haptophytes (*Chrysochromulina parva*), green coccale algae (*Botryococcus*) and dinophytes (*Peridinium willei*), For specific (abundant) cyanobacteria such as *Planktothrix* even a good quantitative relationship between HTS read numbers and *Planktothrix* biovolume was observed.

2) New relevant phytoplankton taxonomic information includes mostly picocyanobacteria, i.e, the genera *Synechococcus* and *Cyanobium* which are hardly detected via microscopical methods. Aside from *Planktothrix* other relevant nuisance cyanobacteria (i.e. *Microcystis aeruginosa, Tychonema bourrellyi, Dolichospermum lemmermannii*) or so far overlooked cyanobacteria (*Prochlorothrix, Nodosilinea*) have been detected.

3) The unequivocal identification of phytoplankton taxa is considered critical for monitoring the toxigenic basis of algal growth. For example, in contrast to *Planktothrix rubescens/agardhii* carrying the microcystin biosynthesis genes, other genera as *Snowella* and the picocyanobacteria composed of *Cyanobium* sp. or *Synechococcus* sp. must be considered less toxigenic as they are less likely to carry the ability to produce microcystins or related compounds.

Fig. 8. Key lake Garda in Italy.

For **phytobenthos** in total 22 rivers were assessed resulting in 53 samples. Two thirds (66%) of river samples had a catchment area > 1000 km2, 23% had catchment area 101-1000 km2, and 9% had a catchment < 50 km2. During sampling, water temperature ranged from 4 to 22°C (10-20°C) and conductivity varied between 25 and 1033 µS/cm (55% of samples had 200-375 µS/cm). To infer overall trophic conditions three nutrient parameters (total phosphorus, phosphate and nitrate concentrations) were used. In general river samples were assigned a trophic state ranging from oligotrophic to mesotrophic conditions (Fig. 9), according to EU Commission staff working document, 2018, Report from the Commission to the Council and the European Parliament on the implementation of Council Directive 91/676/EEC concerning the protection of waters against pollution caused from agricultural sources based on Member State reports for the period 2012-2015 (accessed on 16.6.2021 at https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:52018SC0246&from=EN).

Using 16S rDNA the biodiversity observed for cyanobacteria in river samples was high and was including 25 genera, such as from unicellular cyanobacteria (*Chamaesiphon*), unicellular cyanobacteria forming nanocytes (*Pleurocapsa, Chroococcidiopsis, Aliterella*), filamentous cyanobacteria (*Tychonema, Phormidium, Phormidesmis, Leptolyngbyaceae, Microseira, Wilmottia*) and filamentous cyanobacteria forming heterocysts (Calothrix). The genera *Chamaesiphon, Pleurocapsa, Tychonema* and *Calothrix* occurred with a high number of genotypes (>30). Previous unknown cyanobacteria included (i) the coccale cyanobacterium *Aliterella,* which has been described as a marine deep water or benthic species and (ii) the thin filamentous cyanobacterium genus *Phormidesmis* described from stones in oligotrophic glacial streams or subaerophytic from cold wet rocks. Using 18S rDNA additional reference species (for oligotrophic conditions) detected by sequencing only include red algae (e.g., *Audouinella hermannii, Batrachospermum boryanum*).

In summary it can be stated that for cyanobacteria correspondence between microscopy and 16S rDNA sequencing is useful to confirm microscope based identification of genera. Several previously unknown cyanobacteria have been detected that might require further study (genera *Aliterella, Phormidesmis*). Finally the 16S sequencing information can be useful to infer the toxigenic potential at certain sampling sites, e.g the *Tychonema* genotype Seq No34 which has been detected among river samples but has been linked to anatoxin-a production in the phytoplankton in lakes previously (L. Como, L. Garda (Fig. 8), L. Iseo, L. Ledro, L. Maggiore, Staffelsee Nord).

Using 18S rDNA sequencing in parallel 7 divisions of eukaryotic algae were detected: Chlorophyta (5 classes, most abundant orders Chaetophorales and Ulvales), Streptophyta (4 classes), Cryptophyta (1 class), Dinophyta (1 class), Haptophyta (2 classes), Rhodophyta (4 classes) Ochrophyta (5 classes, most abundant orders Pennales). With regard to genotype and read numbers the Chlorophyta and the Ochrophyta (Bacillariophyceae) were most frequent followed by Rhodophyta and Streptophyta. This additional information derived from 16S and 18S rDNA is considered most relevant, as macroscopic algal growth influences (epiphytic) diatom growth.

Using rbcL the biodiversity was assessed for benthic diatoms only, taking advantage of the curated database differentiating diatom taxa on the level of morphospecies incl morphological varieties (R-Syst::diatom). Since all samples were counted for diatom composition in the microscope (n = 53) in parallel to sequencing (i) a direct comparison as well as (ii) an estimate of the additional information through rbcL sequencing was obtained.

In total 82 diatom taxa were identified through HTS which were detected at least partly also during LM counting. Among the 82 diatom taxa detected by both methods the majority of the taxa (n = 57) were detected more frequently through HTS, ranging from 1.1 - 18 (54)-fold increased frequency of detection (median 3.3 - fold). Vice versa, the minor share of taxa (n = 18) were detected less frequently through HTS, i.e. 0.26 – 0.93 – fold lower detection rate (median 0.54-fold).

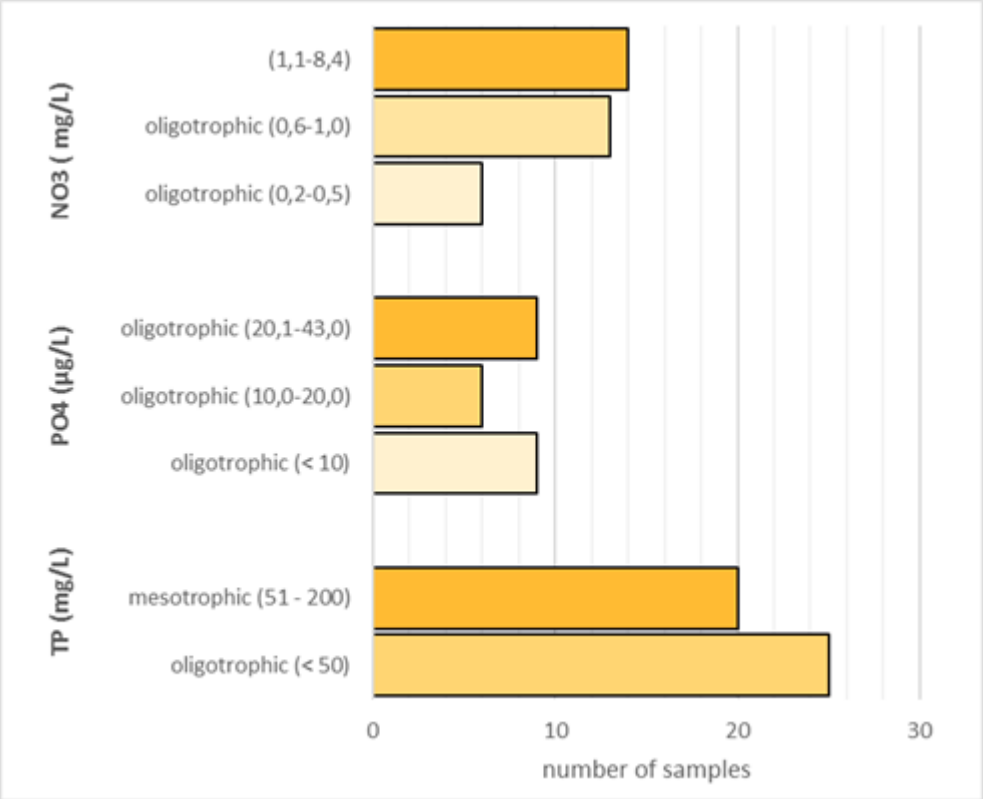


Fig. 9. Characterisation of key river samples from biofilm (BFM) according to (i) TP - total phosphorus concentration exhibit oligotrophic and mesotrophic state, and (ii) PO4 - phosphate and (iii) NO3 - nitrate concentration (classification according to the EU commission working document 2018)

In summary it can be stated that a significant share of diatom taxa was represented by both methods supporting modern microscopy based estimates of diatom biodiversity in rivers. The additional information through HTS can be summarized as follows:

+) HTS analysis supports through confirmation of the microscopy based results and supports diatom morphospecies differentiation in field samples

+) Detection of invasive taxa that cannot be easily differentiated in the microscope such as the differentiation of *Achnanthidium delmontii* in many river samples which is not readily differentiated by light microscopy from the more abundant A. pyrenaicum.

+) in analogy to picosized cyanobacteria and eukaryotic flagellates small sized diatom cells (frustules) are overlooked and probably underestimated in the LM counting procedure, such as *Mayamaea atomus* var *permitis*.

For **fish**, sampling of eDNA in the key lakes (e.g. Figures 10) was done according to the Eco-AlpsWater protocol (D.T1.3.1.). In each lake, multiple 30 L samples were collected along lakeshore transects (6 km each) and at three pelagic locations, including the deepest point of the lake. In Austria and Germany, an additional sampling approach was carried out in which numerous 5 L samples were collected at electrofishing and gillnetting sites that were used during the last traditional assessment.

Standard sampling, carried out by all project partners, included the collection of 30 liters of water along transects (6 km) and filtration through VigiDNA® 0.45 µm filter cartridges using a peristaltic pump. In addition to the shoreline transects, vertical depth-integrated samples were collected at three pelagic sites. The integrated samples were then filtered through a VigiDNA® 0.45-µm filter cartridge. After filtration, the cartridges were filled with a preservation buffer and stored until DNA extraction. In the additional samplings carried out by German and Austrian project partners, 5 L were collected at the same electrofishing and gillnetting sites that were used during the last traditional assessment. Back in the laboratory, the samples were filtered through glass fiber filter discs (GFC) 1.2 µm using a multi-branch filtration system. After filtration, the filters were stored frozen at -20° until DNA extraction.

All the methods used for DNA extraction, sequencing and bioinformatic analyses followed the procedures reported in the brochure 2 (technical guidelines) and at the webpage in the protocol section. More detailed information are present also in the deliverables D.T3.2.1 and D.T3.2.2 (at the webage)

The taxonomic inventories obtained from the bioinformatic analysis were compared to the dataset obtained from the latest traditional fish status assessment for each lake. The traditional methods consisted of pelagic and benthic gillnetting and electrofishing along the shore.

Fig. 10. Key lake Starnberg in Germany.

Comparing the fish biodiversity (as taxa lists) obtained from HTS with that obtained using traditional catching methods, a pronounced heterogeneity among lakes was evident. The effectiveness of the molecular methods to provide a complete picture of fish taxa in a lake (the coverage) might be assessed by the percentage of taxa found with traditional methods only, i.e. not detected with HTS methods. Therefore, the results listed in the Table 6 are ranked accordingly. Best results were obtained for Lake Mondsee were all fish taxa known to live in the lake were detected by HTS, however, only for the samples obtained at the shoreline and filtered with GFC filter discs. For those samples obtained at Mondsee shoreline and open water stations, and filtered with VigiDNA cartridges, the result was considerably less comparable (rank 6 out of 8). In contrast, the two filtering methods yielded almost similar results at Lake Starnberg (ranks 3 and 4) with VigiDNA filtering showing slightly better results. For all other lakes only VigiDNA filtering results were available, for which best results were obtained at Lake Bourget with 96% of fish taxa detected via HTS methods and only 4% not (rank 2). For all other lakes HTS results underestimated fish taxa inventories leaving between 19 % and 58 % of fish taxa undetected by HTS methods. Lowest ranks were found for Lake Garda, although exceptionally some samples using Sterivex filter cartridges were included, and for Lake Lugano were as much as 58% percent of fish taxa were left undetected by HTS methods.

Table 6. Results on comparison between traditional monitoring and HTS. Traditional only: % of taxa found exclusively with traditional methods; HTS only: % of taxa found exclusively with the analysis of eDNA; Both methods: % of taxa found by both methods; the total number of species is reported in the last column.

Waterbody	Filter type	Traditional only	HTS only	Both methods	Total species detected
Lake Mondsee	GFC only	0%	21%	79%	24
Lake Bourget	VigiDNA®	4%	35%	61%	23
Lake Starnberg	VigiDNA®	19%	16%	65%	31
Lake Starnberg	GFC only	21%	17%	62%	29
Lake Bled	VigiDNA®	23%	15%	62%	9
Lake Mondsee	VigiDNA®	33%	10%	57%	21
Lake Garda	VigiDNA®	38%	31%	31%	25
Lake Lugano	VigiDNA®	58%	25%	17%	24

Possible reasons for the heterogeneity among HTS results may include differences in sample collection and downstream analysis. To standardize our investigations as much as possible we adopted a common sampling protocol using VigiDNA cartridges as a “common denominator” across all lakes. Although the analysis of VigiDNA cartridges was centralized and done by a single laboratory (FEM), which was furthermore quality assured by an intercalibration test (Riccioni et al., in revision), results varied extensively. This may be due to slight differences in sample storage conditions and storage time. Specifically, we encountered issues with low DNA yields in a number of samples after VigiDNA storage under cold temperatures as well as microbial growth after weeks to months of storage time. Another factor included unplanned delays in the extraction of DNA caused by laboratory equipment issues. Nevertheless, for the influence of the storage and preservation buffer some questions remained open similar to the findings of the DNAqua consortium, resulting in questionable reproducibility of the preservation and extraction. The additional sampling at two Lakes involving GFC filtration and sequencing in another laboratory yielded heterogeneous results also, with closely matching numbers at Lake Starnberg and quite differing numbers at Lake Mondsee.

Fig. 11. Key river Adige in Italy.

Additional data representative of the individual water bodies (including rivers, e.g. Figure 10) have been described in the deliverables d.t3.2.1 and d.t3.2.2.

In conclusion our experiences involving various filtration methods (mainly VigiDNA and GFC filters, to a lesser extent Sterivex filter cartridges) can be summarized in the Table 7. The raw data collected so far are numerous and still subject to careful examination, which require more in-depth analyses to further evaluate the significance of the results and the comparison of the different approaches.

Table 7: Method comparison, their advantages and disadvantages.

Method	**Advantages**	**Disadvantages**
VigiDNA	Suited for filtration of large volumes of water Allows on site filtration Contamination is less likely	Preservation buffer issues (requires immediate extraction) Labour intensive extraction process higher costs for filter cartridges
Sterivex	Easy handling Allows on site filtration Contamination is unlikely	Only relatively small volume can be filtered leading to low DNA concentration Higher costs for filters
GFC – Glass fiber filter discs Open filtration	Easy handling Suitable for filtration of up to 5 liters	Filtration needs to be done in the laboratory Theoretically prone to contamination

EAW Toolbox - Navigating through the methods and results

(from deliverable D.T3.5.1)

EAW Toolbox for the implementation of innovative monitoring approaches includes the description of all the steps required to integrate the traditional monitoring procedures with methods based on the use of eDNA coupled with HTS to describe the biodiversity of macro- and microorganisms used as indicators within the EU Water Framework Directive (WFD, 2000/60/EC). Previous intercalibration processes have been implemented to ensure a coherent and harmonious implementation of this Directive. Within the EAW project, a wide survey on national methods in the Alpine region identified the gaps and potential implementation of approaches intra and inter countries, providing tools and guidelines for the harmonic integration of HTS-based approaches.

One of the goals was to evaluate and validate up-to-date experimental protocols for metabarcoding to provide useful guidelines for stakeholders and governmental agencies. Through this task, an active network was created that offers flexible tools that can also be used in other contexts. The methods required for the analysis of eDNA coupled with HTS are formalised in several protocols and guidelines that the EAW consortium made freely available (in a form of e-publications) to stakeholders and governmental agencies which can follow the different steps described to integrate the current traditional methods with these up-to-date approaches.

The species list obtained for the different organisms targeted by using the eDNA metabarcoding approaches and using the traditional methods have been uploaded in an Access database in which all the results have been recorded to enable a direct comparison of taxa inventories for each sample. In order to provide easy access to data and metadata, various ready to use queries help to extract information specific for gene markers, genotypes, species or for gap analysis.

Links to most important outcomes of the EAW project are gathered at EAW homage:

(1) Discover key findings in 3 e-Booklets:

- #1 Brochure for wider public
- #2 Technical guidelines for end-users
- #3 Outputs for stakeholders and decision-makers

(2) Explore extensive info-material:

- # lists of all dissemination activities
- # project videos

(3) Download free protocols for eDNA sampling, extraction, library preparation and bioinformatics.

(4) Use our EAW taxa analyses tool and read our FAQ.

(5)See EAW Guidelines and policy recommendation.

(6) Join our EAW Alpine network for the future activities!

Cooperation network description

(from deliverable D.T3.6.2)

I-Defining agreements and areas of collaboration and durability strategy

The effort to provide 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, allowed to create a living network, providing transferable instruments to be applied also in other contexts **(EAW Alpine Network)**. Following EUSALP AG1, the cooperation has been expanded between government agencies and academia, for a mutual exchange of knowledge and creation of a web of connections between project partners, observers, stakeholders and target groups deputed to the assessment of the ecological status of water resources in the Alpine Space area.

Fig. 10. Key lake Bled in Slovenia.

The activities carried out during the implementation of the project, and in particular during the sampling activities, laboratory analysis and data analysis, required the collaboration of various multidisciplinary groups of experts belonging to both academia and government agencies. These continuous collaborations and interactions promoted the transfer of knowledge to the government bodies (such as Environmental agencies) responsible for the implementation of the WFD/WPO. The aim of the "Alpine water cooperation network" (Figure 10) is to promote "a set of guidelines for the adoption of HTS and new biomonitoring tools in WFD by Environmental agencies, improving basin management plans and restoration actions." The consortium is continuously promoting cooperation actions, especially during these last phases of the project. Long-term relationships among the official partners, observers, stakeholders, as well as new persons/bodies are the results of this interdisciplinary network that will ensure the collaboration also after the closure of the project. This is not a new strategy and several forms of "never-ending strategies" have been adopted; a good example is the Cyanocost

network, which was created during the implementation of a COST action, concluded in 2016. CYANOCOST is involved in the risk management of cyanobacteria and cyanotoxins in water bodies across Europe by establishing strong collaborations between academia, authorities, industry and citizens and creating a solid knowledge network. CYANOCOST action aims to provide tools to end-users (public authorities, water utilities, aquacultures, tourism and recreation sectors) by pooling and coordinating expertise throughout Europe and to harmonize methods and practices across Europe, thereby protecting public health, enterprises and investments.
Another example of cooperation presents the active connection with **DNAquaNet COST** action. Members from FEM, NIB, INRAE and SUPSI have been closely involved in DNAquaNet activities by meetings, presentation, ring-test for diatoms metabarcoding, stakeholder workshops etc. In a similar manner, with some of the project partners' participation, we have also established synergies with **SYNAQUA project**, which demonstrated the cross-border synergy of two countries for biomonitoring and preservation of aquatic ecosystems.

Synergies with two Interreg Alpine Space projects were established due to the fact that we have common key water body sites, where experiences have been shared via open communication between specific partners. These two projects were: **HymoCARES** project dealing with Hydro-Morphological assessment and management at basin scale for the Conservation of Alpine Rivers and related Ecosystem Services and the **SPARE** project - Strategic Planning for Alpine River Ecosystems, which aimed at increasing awareness and knowledge about the functions and services healthy rivers are providing and improving river management practices by integrating participatory approaches.

The Alpine water cooperation network (EAW network) is based on formal forms of communication, the most important is a comprehensive mailing list through which experiences and questions can be exchanged and future contacts and project opportunities can be maintained. A first mailing list has been used to disseminate the EAW newsletter: it includes more than 400 people, and it is expected that more will be added during the next dissemination initiatives.

The network is explicitly described in a full dedicated web-page of the project, which sets out the rationale of the network, and invites people to provide feedback on the project`s results and joining the mailing list. In this regard, a dedicated email will be used (ecoalpswater@gmail.com), which is linked to the IT Google Drive platform that is used to exchange information and materials among the EAW partners. The aim of EAW consortium network is to provide coordinated expertise and harmonised methods and guidelines across Europe and lay the foundation for a standardisation of eDNA metabarcoding approaches for biodiversity monitoring in the Alpine region and beyond. Durability strategy with different communication channels is presented graphically on Figure 11 (roots).

II-Cooperation outside the Alpine area

Application of EAW protocols in central Italy

The EAW partner ISPRA (PP9) has promoted and established a working group, in the framework of National System for Environmental Protection (SNPA), involving eleven regional environmental agencies (ARPA; Campania, Emilia Romagna, Friuli Venezia Giulia, Lazio, Liguria, Lombardia, Marche, Puglia, Toscana, Umbria, Valle D'Aosta) and considering water bodies for the extra Alpine experimentation of metabarcoding techniques.

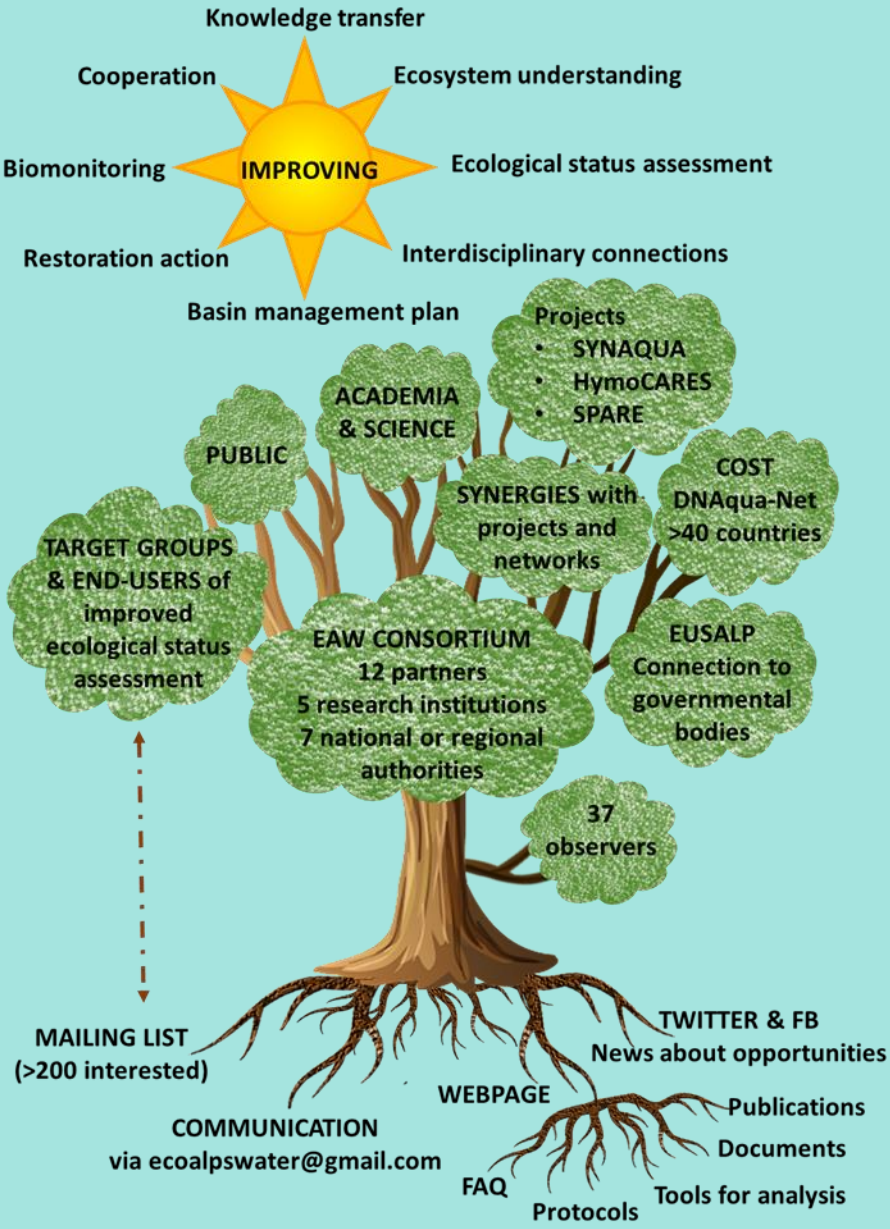


Fig. 11. The Alpine water cooperation network is a web, represented by canopy of connections, which enables interdisciplinary knowledge and transfer of know-how. Alpine water cooperation network is improving important water connected issues, represented by the sun. The roots represent the channels for future cooperation, interactions and durability strategy of Alpine water cooperation network.

The working group met online several times to discuss and verify the feasibility of the protocols developed in the Alpine basins and to select a limited number of informative and diversified water bodies, which originally included lakes and rivers in Lazio, Umbria, Tuscany, and Marche (Table 8).

Table 8. Lakes and rivers initially considered for the extra alpine survey.

LAKE	Vico (Latium)	Trasimeno (Umbria)	-
RIVER	Fibreno (Latium)	Rio Freddo dell'Esino (Marche)	Torrente Stura (Tuscany)

Due to COVID19 pandemic restrictions, it was not possible to extend the experimentation to many of the selected areas and to avoid too many contacts between field operators, only Latium Region's freshwaters were investigated. The experimentation in the Lazio region was conducted in 2021 in four water bodies: the volcanic Lake Albano, the karst Lake Canterno and the Aniene and Fibreno rivers. These sites were selected for their different environmental characteristics, for the high variability of species and for the presence of historical data series collected with traditional methods. Once collected, the water samples were shipped to the sequencing platform at FEM, and then processed with bioinformatic analyses to obtain taxonomic lists of phytoplankton, cyanobacteria, diatoms, and fish. The results have been discussed at two national meetings organized on September 10th and October 13th, 2021. In the near future, ISPRA has planned to promote additional trials in extra Alpine areas with the collaboration of other environmental agencies; at the same time, the experimentation of the SNPA at national level is expected to continue.

Connection with the Long Term Ecological Research (LTER) network

The key element of LTER investigations is their long-term approach, on time scales that go well beyond the typical duration of research projects. Scientific monitoring constitutes a central element of LTER research, including not only the collection of data (basic monitoring), but also their interpretation, modelling and experimental manipulation, with particular attention to key variables in order to identify significant environmental stressors affecting ecosystems, communities, species and populations. This approach necessarily requires to be implemented in a context of scientific research capable of maintaining a high level of quality both in the data collected and in their processing, interpretation, dissemination and enhancement in wider national and international contexts. To maintain a high level of quality in the data collected, the approaches adopted in the LTER investigations must undergo an ongoing review and updating process.

One of the most active field of research in the freshwater, marine, and terrestrial LTER site is focused on the assessment of changes in biodiversity, which is considered as a central element in the functioning of ecosystems. Most of the time, biodiversity inventories are carried out by using traditional approaches, with all the limits that traditional methods have in terms of range of species detected, differences among taxonomic identifications, and replicability. In this regard, the integration of HTS approaches into the traditional biodiversity assessment protocols becomes necessary. This should however, require a process of updating of competences not only within the research institutes, but also within the government offices (e.g. environmental agencies) that are generally directly or indirectly involved in biodiversity investigations. In this process of upgrading, the EAW project played a central role in the integration of novel and traditional methods. In perspective, the EAW Alpine Network will be tasked with maintaining a vibrant community focused on adapting and testing newly developed “omics” technologies to be integrated in routine monitoring plans.

River basin management plan

(from deliverable D.T3.4.1)

The EU Member States should provide river basin management plans (RBMP) about all executed activities every six years to report amongst others about status of water bodies and the success of measures. The required activities and methodology are precisely described in the WFD but not in the RBMP. Particularly the RBMPs describe the execution of the WFD and the success for example in coming closer to the targets. In RBMP there are descriptions of the amount of water bodies in high, good or worse status and which or how many measures are planned to improve the status. On the other side, the methodology to detect biological elements is described in separate papers/instructions or websites and in the technical reports of the intercalibration activities. Therefore, it is meaningful to discuss the links of the Eco-AlpsWater metabarcoding methodology to the requirements of the WFD and to the assessment methods of the Member States, here the five EU-countries and in addition the Switzerland approach (WPO). All of these assessments require taxa inventory lists, which have to be compiled by specific sampling and detection methods to which the metabarcoding approach by the project Eco-AlpsWater can contribute significantly.

Fig. 12. Key river Soča in Slovenia.

Concerning the sampling methods, the metabarcoding approaches by the project Eco-AlpsWater were kept as similar as possible to the WFD methods, so sampling can be done in parallel, or as in case of fish can drastically reduce the sampling effort. The easy and cost-effective eDNA sampling is very useful in large-scale surveys, and to follow up the effect of measures to improve the ecological status with short-time repetition and high spatial resolution, which is not feasible with traditional methods.

The future prospects for implementation of the EAW innovative monitoring approaches into RBMP are:

- Combination of traditional and eDNA approaches allows biodiversity assessment at an unprecedented level.
- Cost efficient eDNA approaches are perfectly suited for large-scale, continuously repeated monitoring, providing the ability to detect changes in the ecosystem at an early stage and to react accordingly.
- Development of eDNA metrics, especially for questions exceeding WFD/WPO, e.g. climate change or control of effects of measures to improve the status.

For bio-components, which are not covered at all so far with national traditional methods (e.g. phytobenthos without diatoms) there is a high potential to use the metabarcoding approach since taxa coverage is already high enough that an eDNA bases metric can be developed. An example of key rivers on Figures 12 and 13.

In perspectives, new metrics provided by innovative eDNA methods can be used also to complement indicators for the study of ecosystem functions and services. However, this is an open and exciting field of research that can rely on the use of all the biodiversity dimensions, which traditional methods cannot determine. To exemplify, this includes the determination of organisms difficult or impossible to determine by isolation and culturing methods, or organisms requiring absolutely exaggerated analysis times compared to the rapid results required by metabarcoding biomonitoring (such as bacteria, pico-cyanobacteria, small micro-eukaryotes). Perspectives for the development of new metrics are discussed in the next chapter.

Fig. 13. Key river Steyr in Austria.

Ecosystem proxies

(from deliverable D.T1.4)

The concept of ecosystem services has grown in popularity over the last decade, however, the value of the full set of ecosystem services provided by lakes, in particular for non-commercial services such as biodiversity, are still not completely examined. Here we did not attempt a detailed assessment of ecosystem services provided by large lakes (EAW sites) which would go far beyond the scope of the EAW project and which would require extensive new studies to be performed. Our goal is to emphasise how metrics related to biodiversity and, in particular new metrics provided by innovative eDNA methods, could be used to complement indicators for the study of ecosystem functions and services. We provide a synthetic Table 9 presenting the main services for which biological metrics (whether traditional or eDNA methods) could be mobilised and indicating the potential benefit of eDNA tools.

I-Ecosystem services & lakes

Ecosystem services are the benefits that humans derive from ecosystems. In the Millennium Ecosystem Assessment (MA 2005), these services were classified in different categories: provisioning, regulating, supporting and cultural services.

Freshwaters (e.g. Figures 14 and 15) had critical importance to humans through history, providing extraordinary benefits to people. In spite of their past and continued obvious importance to human culture and wellbeing, a systematic examination of how humans benefit from large lakes has not yet been undertaken.

Fig. 14. Key lake Starnberg in Germany.

The Common International Classification of Ecosystem Services lists 90 contributions that ecosystems make to human wellbeing (CICES, v5.1). Since the MA, the concept of ecosystem services (ES) has grown in popularity. For scientists, the uptake of ES science is driven by the thought that ES can lead to better environmental decision-making, enabling to understand the role of ecosystems in the provision and regulation services, and to forecast how management as well as local & global pressures might affect the provision of services in the future (Bennet 2017). However, there are still key ES research gaps that are impairing our ability to use ES science to improve decision-making, this includes in particular the lack of indicators and data for numerous services, the need for models linking biodiversity to ecological functions and services, the need for methods allowing cross-scale analysis (Bennet 2017).

Ecosystem services provided by freshwaters are diverse. Sterner et al. (2020) reported that 58 of the 90 CICES services are theoretically applicable to lakes. These authors have described the quantifiable ecosystem services of a set of large lakes and have been able to obtain sufficient data to assess five of these 58 CICES services, mainly within the categories of provisioning and cultural services.

The ecosystem services from large lakes have also been studied in detail for the Laurentian Great Lakes (e.g. Marbek 2010; Allan et al. 2015). However, even in these detailed studies, data gaps in space and time still exist, and, more generally, regulating and non-material services were poorly described due to the lack of indicators. Sterner et al. (2020) also pointed out that more work is needed to show the value of the full set of ecosystem services provided by lakes, in particular for non-commercial services that could not be examined due to lack of data.

Fig. 15. Key lake Bourget in France.

II-Biological parameters in the assessment of functions and services

Biodiversity is to be included in ecosystem assessments in various ways. Mace et al. (2012) reported the key roles played by biodiversity at all levels of the ecosystem service hierarchy: as a regulator of underpinning ecosystem processes, as a final ecosystem service and as a good that is subject to valuation. However, biodiversity (as also scenic and cultural values) is more difficult to monetise or even quantify; as a consequence scientists have not yet completely solved how to treat biodiversity in the concept and practical application of ES studies.

The biodiversity provided by lakes can be measured in terms of genetic, species, populations, communities, functional group, and food-web diversity. Aquatic ecosystems are indeed shaped by the interaction of biological communities with the abiotic environment. Biodiversity and biotic interactions play a key role in maintaining basic ecosystem processes and in supporting ecosystem functions (production, regulation of pollutants, etc).
Schallenberg et al. (2013) compiled information on specific ecosystem services that are the results of lake ecological functioning, thus including the role of species, communities, habitats and ecological processes that are generally less well recognised in ecosystem service studies. Schallenberg et al. (2013) also proposed to divide lake ecosystem services into four types: (i) services that are globally recognised via treaty obligations, (ii) services that provide resources directly, (iii) services that support and regulate useful ecosystem processes and components, and (iv) services that are culturally important. We have adopted this classification here, which fits well to large peri-alpine lakes.

In the EAW project we did not attempt a detailed assessment of ecosystem services provided by large lakes (6 key sites of the EAW project), or an economic valuation analysis of these services, which would go far beyond the scope of the EAW program and which would require extensive new studies to be performed. **Our goal is more particularly to emphasise how metrics related to biodiversity and more particularly new metrics provided by innovative eDNA methods could be used to complement indicators used in SE science.**

Definitions (modified from Bennet, 2017)

- Ecosystem function: An interaction among organisms and their ecosystem(s) that underpin the ability of an ecosystem to provide ecosystem services.
- Ecosystem service: An ecosystem function from which humans can derive benefits, often through additional inputs of other forms of capital.
- Ecosystem process: Changes in the stocks and/or flows of materials in an ecosystem, resulting from interactions among organisms and with their physical-chemical environment.
- Benefit: in this context, a general term to denote the many ways that human wellbeing is enhanced through the processes and functions of ecosystems via ecosystem services.

III-Lake biodiversity metrics and ecological functioning, mobilised in ES science

Biodiversity is a single word that is used for a complex set of measures and concepts. As defined by the Convention on Biological Diversity (https://www.cbd.int/convention/articles) biodiversity is the variability among living organisms from all sources and ecological complexes of which they are part; including diversity within species, between species and of ecosystems. In our exploration of metrics usable for the evaluation of functions and services, we integrate two types of values of biodiversity:

The first one is related to the functional role of biodiversity, and in practice it is not represented a single measurement but integrate various dimensions and biological indicators related to structural and functional biodiversity of lakes. It is well recognised that structural biodiversity enhances the efficiency of ecological processes such as primary production and decomposition (important determinants for water quality). It has been shown that biodiversity (seen as richness and equitability) may help to buffer natural ecosystems against the ecological impacts of nutrient pollution (Cardinale 2011). In this case, the biological metrics to take into account are: genetic diversity, species/taxonomic richness, diversity of biotic interactions in food webs & networks. Functional diversity, which is the variation in the expression of functional traits, is another important determinant of ecosystem functioning. Functional traits help to define species in terms of their ecological roles (i.e. how they interact with the environment and with other species). In this case, the biological metrics to take into account are: body size structure (for species or assemblages i.e. pico-,nano- or micro-plankton), production of toxins, etc.

The second one is not based on its functional role in ecosystem processes, but biodiversity itself is an ecosystem service due to its intrinsic value; generally, in this case organisms have value that is by definition unquantifiable and therefore non-transactable. When biodiversity itself is seen as a service, it is particular biological groups, often charismatic ones, whose conservation is sought. Nevertheless, biodiversity has an existential value for many people who wish to preserve it, irrespective of any direct benefits they derive from it. In this case, biological metrics to be taken into account are for instance the durable presence of emblematic species (endemic, endangered fish …).

IV-Biological indicators from traditional and innovative eDNA tools

The Water Framework Directive (WFD, 2000/60/EC) has been published in 2000 and its implementation led to a new paradigm in the understanding of ecological status of water bodies in Europe. The Directive explicitly requires that the ecological status is assessed through the analysis of various characteristics of aquatic flora and fauna, i.e. different biological quality elements and their metrics. For lakes these are composition, abundance of phytoplankton; composition and abundance of macrophytes and phytobenthos; composition, abundance and diversity of benthic invertebrates; composition and abundance of fish fauna.

More recently, the analysis of environmental DNA (eDNA) by HTS or using other quantitative methods (as qPCR or ddPCR) has been recognized as very promising tool for the evaluation of freshwater biodiversity (e.g. Pawlowski et al. 2018). The eDNA-based methods can be applied to a wide range of taxonomic groups, and diverse type of biological matrices (water, biofilm, sediment), and offer the possibility to inventory a large diversity of taxa within complex biological assemblages, or to target species of interest (or functional genes of interests). The applications are very diverse for both micro- and macro-organisms. The use of eDNA–based methods can significantly improve biodiversity monitoring surveys through the early detection of exotic species, the tracking of elusive endangered species (e.g. Deiner et al. 2018; Taberlet et al. 2018). But this is also important to keep in mind that microbiologists have a large panel of DNA tools that can be mobilized for environmental survey to track the presence of pathogens, functional genes involved in key biogeochemical processes, etc. Overall, reduced costs per sample (for HTS in particular) allow flexibility in scaling up the number of samples and replicates, the temporal and spatial

sampling frequency. The EAW project has allowed the consolidation of various eDNA protocols applicable for some bioindicators used in the WFD.

The metrics that are used to assess the health or state of aquatic ecosystems (whether traditional methods or new eDNA methods) **can also be used to assess the potential of ecosystems to support functions and services** (Palmer & Febria 2012). Here, considering both traditional and new eDNA biomonitoring methods, **we try to link the structural or functional metrics provided by environmental surveys to key ecosystem functions and services** (in lakes).

V-What kind of aquatic organisms are important for functions and services in lakes?

According to Mace et al. (2012), all the biodiversity components, determine the quantity, quality and reliability of ecosystem services. Here we inventory the main biological groups that are of interest in the evaluation of functions and services (in lakes). Though the list is probably not complete, it illustrates that all types of organisms, from micro-organisms to macro-organisms, can be linked to important functions in lakes. The benefits obtained for human populations are diverse: clean water, food production (fish), contribution to the regulation of nutrients and chemical cycles, potential regulation of some contaminants, conservation of genetic variability required for the adaptive potential and preservation of protected species. In addition, healthy ecosystems provide cultural- and recreational services, aesthetic enjoyment, education and also biological compounds, potentially of interest for the development of novel pharmaceuticals.

The main biological groups or biodiversity dimensions to take into consideration in such ES evaluation are :

- Microorganisms (bacteria and fungi) involved in decomposition and nutrient cycling
- Primary producers (including micro-organisms, plankton) involved in biomass production and carbon capture
- Top predators and parasites that are key actors in populations' regulation
- Genetic diversity (for diverse biological groups) to ensure resilience of the systems against future climate change/diseases
- Large vertebrates, fish, birds which are emblematic, charismatic and of aesthetic interest
- Key species or umbrella species, that provides 'protection' for wider communities, or play key roles in the equilibrium of food webs and habitats
- Phylogenetically distinct species that allow evolutionary diversity to be maintained
- Endemic and/or patrimonial, charismatic species for maintaining macro-organisms diversity
- Endangered species for maintaining taxonomic diversity
- Organisms able to produce secondary compounds offering a potential for commercial use, as for instance novel pharmaceuticals

Table 9. The main ES for which biological components play a role, the biological metrics, which are informative for these functions and services and the potential benefit of eDNA methods.

ES Type Provisioning				
Benefits	**Conditions favouring species, community or process of interest**	**Main pressures involved**	**Biological indicators**	**Potential added value of eDNA**
Drinking water	For maintenance of good quality of water (drinking purpose): -No cyanobacterial blooms and toxic micro-algae - No bacterial pathogens (for humans) -Low contaminant levels -Low turbidity	-Eutrophication -Climate change -Pollutants	-Cyanobacterial community composition and biomass -Presence of toxins -Presence of active genes involved in degradation of contaminant -Presence of pathogens (to human)	- Potential toxicity: early detection of genes involved in toxin production (ARN for active cells) -Possibility to detect active genes (transcripts) involved in contaminants degradation - Detection of rare signals, notably for pathogens (bacteria) - Extension of spatial & temporal capacity of survey
Fisheries (salmonids & perch) Recreation & tourism Salmonids and other sports fish + Birds	For maintenance of good quality of noble fishes : -Low to moderate nutrient enrichment -Low contaminant levels (e.g. PCB) -high dissolved oxygen -Cool temperature - Habitats for the different stages of fish life cycle	-Eutrophication -Climate change -Modification in habitats and/or spawning sites -Intensive fisheries -Invasive species -Fish pathogens	- Fish biomass for exploited fish (e.g. perch large lakes) -Diversity in the composition of Fish assemblages - Maintenance of salmonids & relative proportion of salmonids - Genetic structure of biological populations	-Extension of spatio temporal scale for monitoring of fish assemblages -Detection of rare species, -Detection of fish pathogens, -Early detection of invasive species -Monitoring of phenology (in spawning area for instance, using targeted eDNA approaches).
Genetic materials from all biota	-Low to moderate nutrient enrichment -Low contaminant levels - Maintenance of diverse habitats	-Eutrophication -Climate change -Invasive species	-Estimation of genetic diversity -Genetic structure of biological populations	- Expand the range of biological groups considered - Possibility to reveal different levels of diversity (e.g. infra-specific diversity)

ES Type : Regulation & maintenance				
Benefits	**Conditions favouring species, community or process of interest**	**Main pressures involved**	**Biological indicators**	**Potential added value of eDNA**
Mediation of nutrients/ Pollutants/ wastes by biota	-Removal of reactive nitrogen compounds and excess of nitrogen (denitrification, anamox, etc) -Long-term sequestration of nutrients in sediments -Nutrients uptake (by macrophytes) -filtering water by filter feeders (e.g. daphnia) -Removal of reactive nitrogen compounds and excess of nitrogen (denitrification, etc)	-Eutrophication -Local pollution : ancient and emerging organic pollutants, … -Climate change -Modification of oxic-anoxic boundaries	-Abundance and activity of bacteria and archae involved in biogeochemical processes (e.g. denitrification) and degradation of pollutants -Bacterial production involved in decomposition of organic matter -Phytoplankton biomass and composition, Primary production, [chla] diatoms composition => quality indices -Composition and biomass of filter feeders (Daphnia)	-Quantification of genes involved in nitrogen transformations (denitrification, anamox, methanotrophy, …) -Quantification of active genes involved in degradation of pollutants -Early detection of potential toxicity (genes toxin) -Extension of spatial & temporal scale for biomonitoring for diverse issues
Biodiversity	For maintenance of high levels of biodiversity : -Low to moderate nutrient enrichment -Low contaminant levels - Maintenance of diverse habitats	-Eutrophication -Pollutants -Climate change & Increased hypoxia -Invasive species	Metrics on diverse biological groups : bacteria cyanobacteria, micro eukaryotes , invertebrates, fish, birds … and on different compartments of the lake : littoral, pelagic, benthic zones	-Extension of spatial & temporal scale for biomonitoring within the lake -Extension of the biological groups considered in the inventories of biodiversity -Highlight cryptic diversity -Detection of rare signal/species -Access to ecological networks (food web structure) -Early detection of invasive species

Life cycle maintenance	For maintenance of nursery and habitats for fish (or other organism): -Adapted quality of water - Maintenance of diverse habitats -Low to moderate predation	-Modification in habitats and/or spawning sites -Hydrological changes -Climate change -Eutrophication -O2 levels	Spawning activity: -Phenology and success -Abundance of juveniles in nursery zones and survival -Age structure of the population -Production/year of species of interest	-Detection of species at high spatial and temporal scale -Quantitative eDNA detection of species (e.g. qPCR) on specific zones as spawning area -Analyses of intra-specific diversity of populations
ES Type : Cultural				
Benefits	**Conditions favouring species, community or process of interest**	**Main pressures involved**	**Biological indicators**	**Potential added value of eDNA**
Support for scientific investigation Educational interests	NA	Potentially all pressures	Potentially all biological indicators that could serve educational actions	Potentially all eDNA indicators that could serve educational actions
Symbolic and/or endangered species Spiritual aesthetic value Recreation and tourism :	-Low to moderate nutrient enrichment -Low levels of contaminants -Low level of cyanobacteria (filamentous and colonies) -Low turbidity	-Eutrophication -Degradation of habitats -Turbidity -Climate change - Modification of hydrology -Invasive species	-Presence of patrimonial species (fish, birds) -Composition of fish and birds assemblages -Phytoplankton biomass- -Chlorophyll a concentration -Low level of cyanobacteria -Composition of vegetation (macrophytes)	-Early detection of invasive species - Early detection of potential toxicity associated to algal blooms -Early detection of bacterial pathogens -Extension of the biological groups considered in the inventories of biodiversity to give an enlarged view on biodiversity

VI-Conclusion

Here we suggest considering new information and metrics made accessible via eDNA methods; however, the combination of measurements that should be used to evaluate ecosystems functions and services is still a matter of scientific debate. Several questions need to be addressed; for instance, regarding the metrics that can be used as early indicators of degradation or recovery: How do they vary with each stressor? More data on how ecosystem structure and function vary across a large range of conditions are still needed to efficiently determine which set of metrics, under which contexts, best equates to functions and services we expect from lake ecosystems. Beyond the economic paradigm that is inherent to the ES approach, spiritual, cultural and scenic values, which are less often discussed (and quantified), are nevertheless equally important to human well-being.

Guidelines for digital accessing of data

(from deliverable D.T4.1.1)

Guidelines must reflect the interests of the stakeholders working in the field of freshwater monitoring. These were collected during regional, national and international meetings organized by the EAW project, but also by projects with similar scopes.
Stakeholders have raised three fields of interest to the project results:

- Degree of similarity when comparing taxa inventories gained by traditional methods (EU-WFD, WHO-CH) and by metabarcoding approach
- Rating the applicability of metabarcoding approach in terms of cost, practical handling and processing and in terms of assessing the ecological quality of a water body
- Which additional and supporting information the metabarcoding approach can provide

Each field of interest needs an adopted format of digital access for stakeholders. Following examples illustrate different data needs:

- When checking the similarity of taxa identified by traditional and molecular methods, users focus only on taxa relevant for traditional water assessment: they have fixed target groups and indicator taxa in mind when comparing taxa inventories for each sample or water body. Ideally, all data are in a specialized tool and can be selectable “on demand” from the entire and huge EAW data set.
- In case of evaluation of the index results by national assessment metrics the high diversification of metrics must be realized. Detailed metadata are needed to understand which metric was used, and how taxa inventories influence metric results.
- In case of any additive and supporting information provided by the metabarcoding approach the main focus is on structural biodiversity, which enhances the efficiency of ecological processes such as primary production and decomposition (important determinants for water quality). Biodiversity (seen as richness and equitability) may help to buffer natural ecosystems against the ecological impacts of nutrient pollution (e.g. document at webpage). Of further value are, for instance, records about presence of emblematic species (endemic, endangered fish), and of the geographical distribution of specific taxa.

I-How complex are the data collected in the project?

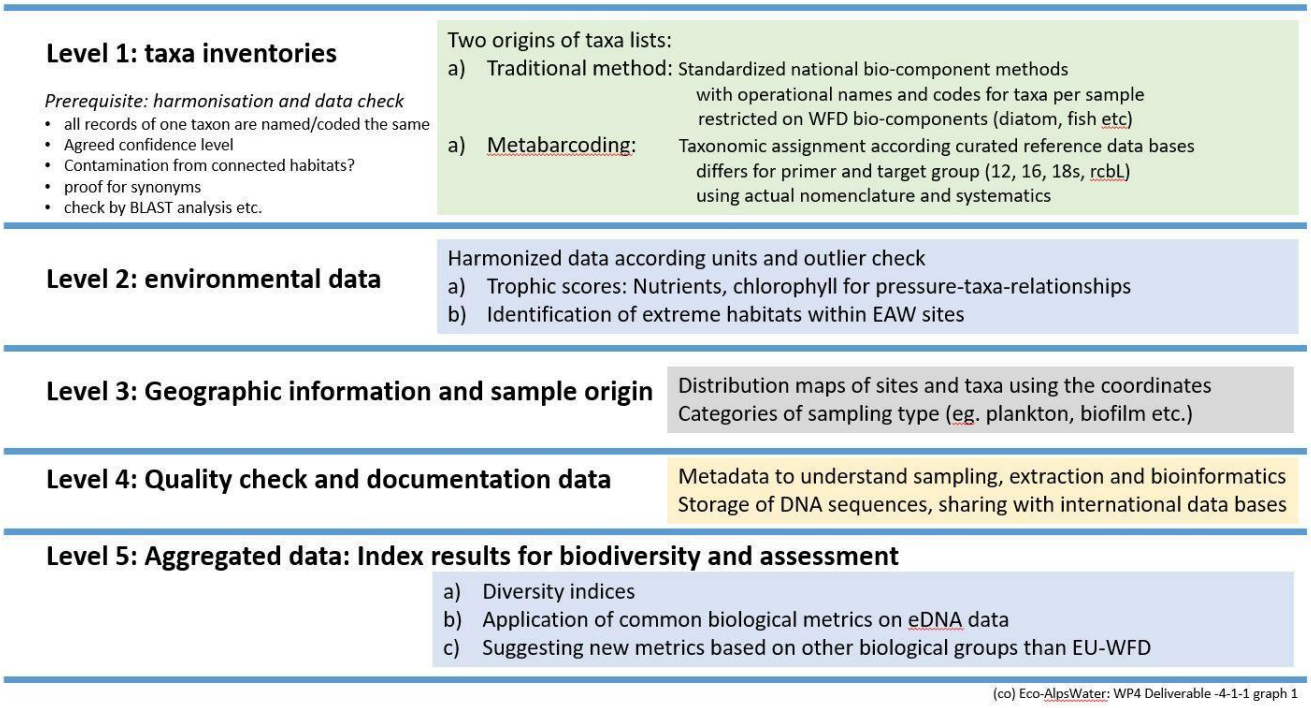
The obtained raw data are very complex especially for the metagenomic results and involve a large number of samples and analysis steps. When focusing on microorganisms (phytoplankton, diatoms, bacteria and fungi) 153 plankton and 177 biofilm samples from 37 lakes and 53 river sites were assessed with multiplex primers.

For each of the three primers, large HTS record tables were produced using the proposed EAW bioinformatic pipelines. These large tables combine all samples with all detected sequences and give the detected signal (rarefied read counts) in the cross field.

The impressive numbers are presented below:

- 11.468 ASVs sequences of the 18S rRNA gene, classified using the Protist Ribosomal Reference database-PR2 (all eukaryotes, including phytoplankton)
- 37.530 ASVs sequences of the 16S rRNA gene, classified using Silva 138 (bacteria)
- 1.285 ASV sequences of "chloroplast 16S" (some eukaryotes)
- 1.602 ASV sequences of rbcL selected for diatoms by library database Diat.barcode were detected and listed for each sample.

The following scheme illustrate the various levels of obtained data:



II-Tool for comparing taxa inventories

The most frequent interest by stakeholders was to compare the metabarcoding taxa list with the records monitored for their national metrics. To explore the taxa inventories, a tool for easy extraction of data is necessary. The "EAW taxa analysis tool" executes analysis steps on demand and automatically. The tool is also a feed-back to user questions which were identified during the preliminary regional data analysis by the project partners.

How to handle a taxonomic output, which leads seldom to a species name, but mainly to higher taxonomic levels?

The HTS record tables provide the taxonomy of a sequence in case there was a match to a reference database (such as the NCBI-GenBank). This match is connected to a taxonomic level such as order, family, genus and species. It is notable that only a small part of all sequences are detected at the species or genus rank. Therefore, the tool delivers separate match analysis on genus or on species level. This is relevant for water assessment since indicator lists in biological WFD metrics mainly contain taxa at the genus or species level.

How to deal with a species that is listed under several sequences (ASVs) in the metabarcoding output?

An important question from users and stakeholders concerned the detection of one unique individual species name under different ASVs. Since the sequences of each ASVs differ at least by one nucleotide, this diversity is due to the presence of several "genotypes" (or oligotypes) of one species. In the case of several sequences belonging to a unique genus, those sequences represent a mixture of different species and/or oligotypes of one unique species, depending on the nucleotide percentage identity. Still, a user of the data just wanted to know if a species or genus is present or not. The tool therefore brings all sequences together, which belong to the same taxon and aggregate the result in one "present" record.

I see an output list from metabarcoding with many taxon names, which I never heard before. How to select and recognize my target taxa?

With the aim of conclusively comparing the taxa inventories obtained by traditional methods (EU-WFD, WPO-CH) and by the metabarcoding approach, the focus is on the biological target groups, the so-called bio-components such as phytoplankton including cyanobacteria and benthic diatoms. The user needs help to separate taxa classes relevant for its target group from all the other organisms groups, which the gene marker might also detect (e.g. trees, mammals etc.). Furthermore, the metabarcoding outputs contain up-to-date names for taxa and classes, which may be different from what the stakeholders are used to knowing from their biological check lists of WFD/WPO monitoring. For comparing lists from HTS and traditional methods, both lists must be harmonized by translating into common taxa names, and here we use those provided by the platform www.freshwaterecology.info, further checked using AlgaeBase (which is also considered by NCBI, from which the two databases, SILVA and PR2, used in the classification of ASVs, retrieve their raw data).

Out of all ASVs and taxa found with HTS, only a small part belong to the target groups and their classes. Therefore, a "phytoplankton filter" helps to select phytoplankton taxa within the 18S taxonomy and a "cyanobacteria filter" to select within the 16S taxonomy. These filters are part of the "EAW taxa analysis tool" and a product of the data preparation. In the case of the *rbcL* marker gene, it is already specific for diatoms. Applying all the selection and aggregation steps mentioned above programmed in the EAW taxa analysis tool, the numbers of detectable taxa within the three target groups are:

- 16S ASVs - 88 detectable cyanobacteria species/genera
- 18S ASVs - 882 detectable phytoplankton species/genera
- rbcL ASVs - 226 detectable benthic diatoms species/genera

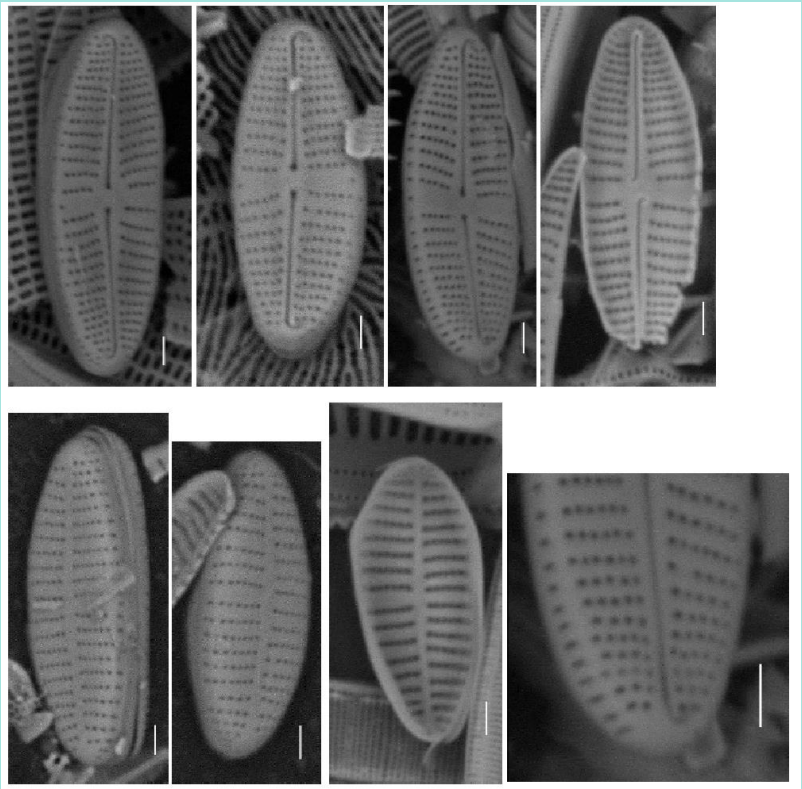


Fig. 16. Confirmation of the eDNA detection of the neophyte Achnanthidium delmontii in pilot river Wertach, DE by scanning electron microscopy (SEM) documentation (Goos, 2021).

Based on the high sensitivity of the metabarcoding approach, neobiotic and cryptic species can be detected, even if they occur in low quantities, and therefore contributing to update present monitoring surveys. A few of these new records correspond to identifications made by using the traditional method, but others do not. Among diatoms, the new species were confirmed by scanning electron microscopy (SEM, e.g. Figure 16). As for fish, identification of new species using traditional approaches can be attempted by intensifying sampling campaigns. Fish taxa lists obtained from integrated samples using VigiDNA-cartridges and the 12S-marker metabarcoding approach were almost complete and additionally revealed the presence of new taxa, when compared to long-time fishery results. There are hundreds of additional genotypes found with an up-to-now undetermined taxonomy from the family to the order levels, which can be explored in the near future.

Is the metabarcoding approach applicable in monitoring in terms of cost, practical handling and water body assessment?

The project partners learned that a close partnership between public water monitoring authorities with experienced scientists is the best key to manage sampling and data analysis with the metabarcoding approach. The costs for the 2 and up to 3 marker runs per sample were comparably low. The sampling effort for eDNA-samples was much less (fish) or equivalent (biofilm, plankton) to traditional sampling. The protocols developed in the project were easy to implement and were standardized.

Which additive and supporting information does the metabarcoding approach provide?

The pilot lakes and rivers studied with EAW metabarcoding approach showed the diversity of various biological groups and communities at an unprecedented level (e.g. Figure 17). For example, the 18S marker detected 54 different ciliate taxa in the plankton samples that would otherwise have been overlooked. Additionally, neophytes such as potentially toxin-producing cyanobacteria were detected (e.g. *Tychonema,* Figure 18).

Fiig.17. Examples for the morphological diversity of phytoplankton in Bavarian lakes.

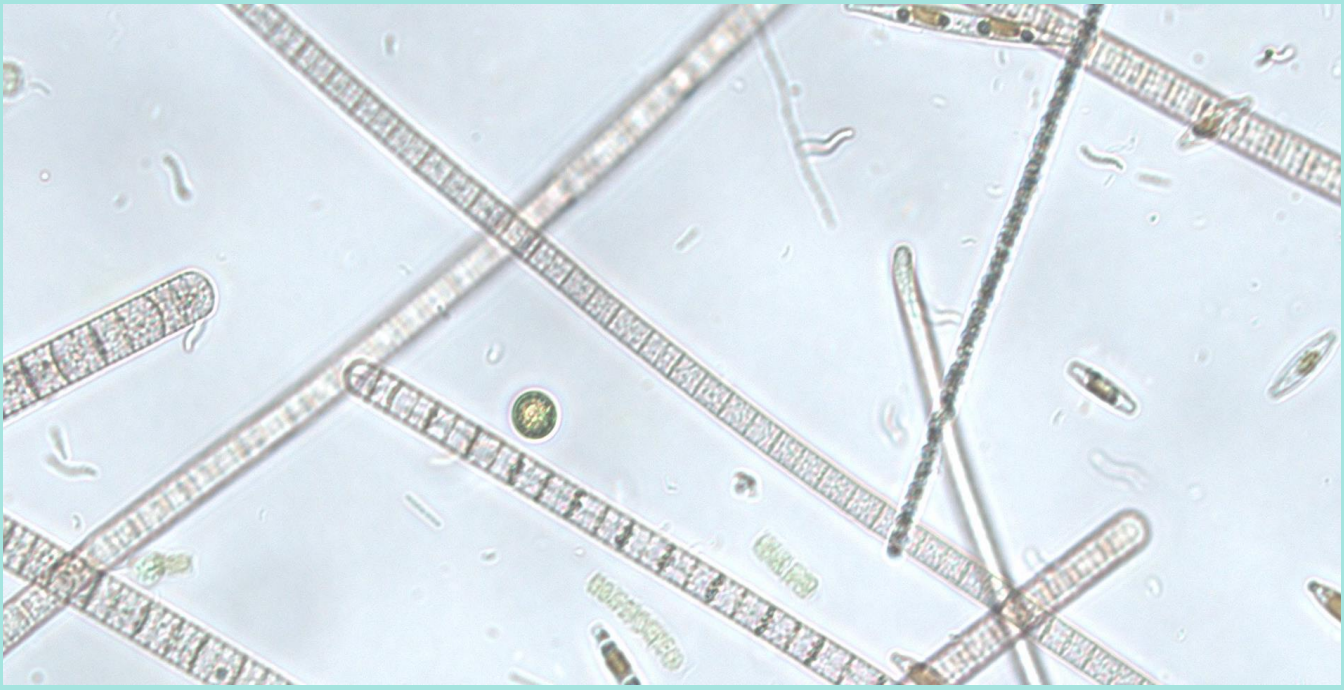


Fig.18. Tychonema trichomes taken from the river lake Mandico in the Lech river system. Determination were confirmed by eDNA (2019/08/30; EAW biofilm sample).

Policy recommendations

I-The innovative monitoring in water quality assessment and management

To fulfil the requirements of the WFD/WPO a biological monitoring is based on the assessment of taxa inventories in freshwaters. They are not only essential to WFD/WPO, but also of great use for other topics in ecosystem analysis and water management (e.g. Figure 19).

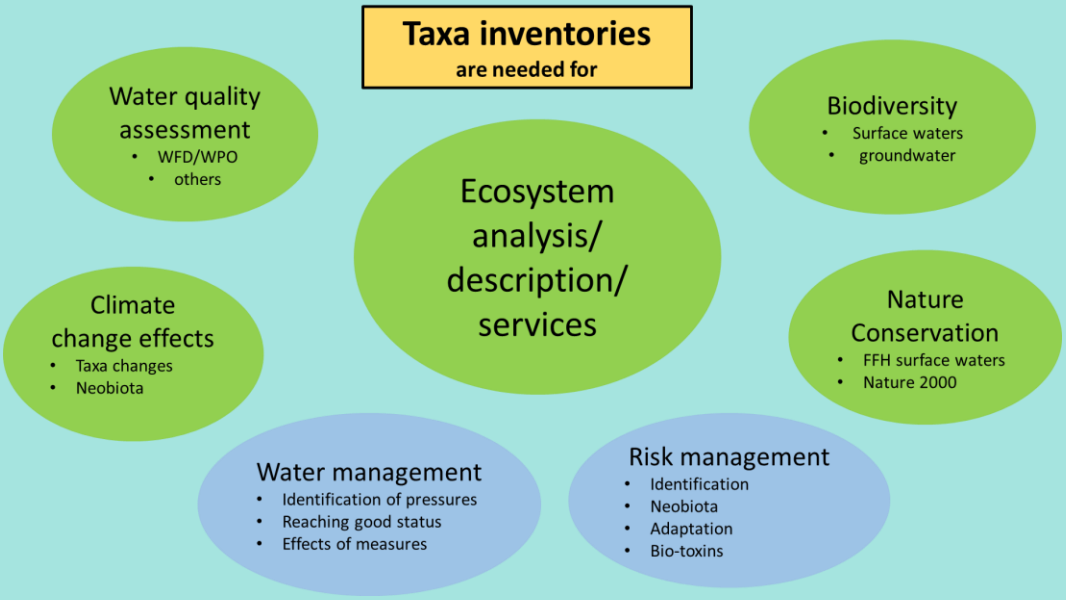


Fig. 19: Illustration of public interests in inland waters to which taxa inventories are relevant.

The new molecular based approach developed by the EAW project and associated results contribute to a decisive improvement in future monitoring of biological quality elements. Traditional monitoring methods have many known limitations, such as

- Difficulties in the determination of certain indicator taxa.
- Difficulties in finding rare taxa.
- Selectivity of traditional methods regarding certain taxa.
- Traditional taxonomy studies, based on the isolation and cultivation of species are rarefying, and most of the time difficult or impossible with species refractory to isolation and cultivation.

The EAW methods are already able to overcome some of these limitations and offer the possibility to answer additional, previously unaddressed, questions. Especially the taxa determination as the main normative element of the required ”composition“ will be supported and improved considerably using the EAW HTS methods. At present, the DNA metabarcoding of benthic diatoms is very promising (e.g. document at webpage).

II-New knowledge in applicability of metabarcoding approach

Stakeholders are interested to evaluate the applicability of the metabarcoding approach in terms of costs, practical handling and processing. What have we learned about it? We can conclude that the cost for the

sampling and analysis of one environmental sample was less (or much less) than that required for the traditional analysis by light microscopy or by fish capture and handling. An important project outcome is that sampling protocols have been harmonised, tested and improved and are now publicly available at project website. The biofilm protocols even became technical reports to CEN.

Sampling and eDNA extraction protocols turned out to be easy to handle and are no obstacle for routine monitoring. The protocols were successfully applied not only at the pilot sites (Figs. 3) but also at many additional sites. Alpine freshwaters frequently show an extremely low plankton content, but the sterile filters proved to be very sensitive in terms of taxa composition.

It is also remarkable that no contamination and no cross contamination in the EAW sample sets were detected, and all blind samples were blank (signals below detection criteria). In case of sampling eDNA of fishes, the newly applied "VigiDNA system" is very time effective. However, the buying process of suitable large volume filters and its storage until sequencing is still of special vulnerability for DNA degradation and have to be optimized. For this reason, other two additional/complementary approaches have been proposed and applied by the EAW partners, based on the use of encapsulated 0.45 µm Sterivex filters and "open" GFC 1.2 µm nominal glass fiber filters (see brochure 2).

Fig x. Key river Wertach in Germany.

III-Can metabarcoding methods in quality assessment and management fill gaps?

- An homogenous approach for pigmented microbial taxa living in biofilms of freshwater substrata ("phytobenthos") becomes possible with the multi-gen-marker approach. The EU Member States handle this bio-element very differently and do not cover all required groups (e.g. focus on filamentous green algae or only diatoms).

- Fish monitoring, especially in lakes, is very time consuming and expensive. The new HTS approach is non-invasive and allows fish species to be detected without harming them; moreover, it is much more sensitive and cheaper.

HTS metabarcoding already detects microzoobenthos/-plankton by 18S and bacteria by 16S in the plankton and in biofilms. There is a chance to improve this HTS method for pelagic zooplankton and bacteria taxa determination, also by selecting specific couple of primers.

- The use of biological quality elements in the WFD is required in different ways for different types of monitoring. For investigative monitoring no requirements are given to identify unknown pressures. Here, the application of the EAW eDNA metabarcoding approach has the highest prospect to support or even monitor it completely in an effective and efficient way. Furthermore, it can be integrated also for surveillance and operative monitoring programs.
- Water management can be improved by efficient and objective molecular–based tools and metrics able to evaluate the taxonomic composition and changes in biological communities. In turn, this has important implications for a better knowledge-based management of water resources, including lakes and rivers that do not achieve good ecological status. The methods of the Water Framework Directive are often not suited to detect minor changes in the ecosystem in response to recovery or mitigation measures, and lack sensitive and easy-to-use methods.

The identification of neobiota at an early stage, with initially low population levels or hidden status, can be supported by EAW methods due to their higher sensitivity, as well as the identification of the presence of toxic or hazardous species like some cyanobacteria. The EAW methods support the implementation of the bathing water directive. Identification of invasive and/or potentially harmful species can improve risk management policies and protection in reservoirs and lakes used for the supply of drinking water.

FAQ - general and scientific perspective

We have collected questions (FAQ) regarding the metabarcoding approaches during national stakeholder meetings. Here we provide general and scientific answers. FAQ Catalogue also available at our webpage.

Why several primers are used in the EAW metabarcoding approach?

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

Answer in detail: We chose the most commonly applied primers to our target groups after literature study: phytoplankton, benthic diatoms and fish. To identify organisms on species level, very specific primers in connection to specific gen reference databases must be applied to phylogenetic close related groups (see rcbL, 12S). In order to detect all subgroups of different target organisms such as phytoplankton, universal primers rather than specific primers are needed (16S, 18S).

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

Microalgae belong to very different phyla of the evolution tree. Therefore, we used generalist markers to detect the microbial assemblages. These markers revealed many hidden species, but conversely they failed to detect circumscribed groups of traditional indicator species.

Answer in detail: This is due to the marker gene we chose which are generalist markers, regions of 18S and 16S which are designed to explore a large diversity. These markers are interesting to get a comprehensive view of the microbial assemblages but are not optimal to reach a fine resolution for each pigmented group within the very divers microbial assemblages. In the near future further gen markers can be combined with the Eco-AlspWater approach to improve species detection of phytoplankton. The rbcL marker genes are specific to diatoms: the completion of molecular database and the problem of synonymies, or 'sister species' are indeed the main reasons for the discrepancies between results of light microscopy and metabarcoding.

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

Both HTS and light microscopy methods may have detected the same genus, but different species. The EAW taxa analysis tool deliver match tables on genus or on species separately for cyanobacteria and eukaryotes.

Answer in detail: The HTS record tables provide a taxon name of a sequence in case there was a match to a gen bank (see bioinformatic deliveries D-T1.1.3 1-4). Not all sequences in fact lead to a genus or species name but to a family or order; this is especially true for the more universal markers such as 16S (bacteria, fungi) or 18S (eukaryotes). Beside the direct match analysis (1:1) between HTS and traditional method, there can still be a match on the genus level. Therefore, the EAW taxa analysis tool delivers match tables on genus or on species separately for cyanobacteria and eukaryotes. This is relevant for water assessment since indicator lists in biological WFD metrics mainly contain taxa on genus or species level. Sequences (ASVs) which identify only family or order level, are not useful as indicator species in traditional methods.

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

The number of DNA sequences (ASVs), which belong to one species (taxon) is an indicator of the intra-specific (intra-taxon) genetic diversity. The EAW taxa analysis tool brings all sequences together, which belong to the same taxon and aggregate the result in one "present" record.

Answer in detail: It is an important information for users and stakeholders that one taxon can be detected with several ASVs. Since the DNA sequence of each ASV differs from each other, this diversity represents "genotypes" of one species. Still, a user of the data just want to know, if a species or genus is present or not. The tool therefore brings all sequences together, which belong to the same taxon and aggregate the result in one "present" record. The number of ASVs, which belong to one taxon, is the intra-specific genetic diversity found a the selected site.

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 EAW taxa analysis tool to compare the lists.

Answer in detail: HTS taxonomy (NCBI) in metabarcoding lists is up-to-date, and thus, many biological names maybe new for the user. With the interest to compare the taxa inventories gained by traditional methods (EU-WFD, WTO-CH) and by the metabarcoding approach the focus is on the biological target groups, the so-called bio-components of the WFD, phytoplankton including cyanobacteria, benthic diatoms and fish. While the harmonisation of names in fish lists is less problematic, the systematic and nomenclature of microorganisms are undergoing permanent changes in very short time. Operational taxa lists for biomonitoring are often more conservative since determination keys and assessment tools are adapted with delay to new phylogenetic findings. In biomonitoring the taxa names are frequently synonyms of the actual taxa names and they are associated with old-fashion systematics.

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

By the term »environmental DNA« (eDNA) we mean the entire hereditary material of all organisms that are (or have been) present in a certain 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). For larger organisms (e.g. fish or humans), it is transmitted to the environment through body secretions, dead skin or hair and can be stored in the form of free 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 or 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.

Answer in detail: By the term »environmental DNA« (eDNA) we mean the entire hereditary material of all organisms that are (or have been) present in this 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). For larger organisms (e.g. fish or humans), it is transmitted to the environment through body secretions, dead skin or hair and can be stored in the form of free 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 or 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 paleo-ecological research. The resistance of dormant cysts of many algal species is very long.

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.

Answer in detail: You can find related projects with advanced metabarcoding methods focusing on these groups. Since we wanted to test innovative approaches, we focused on other biological groupsthat were less well tested. Due to the financial constraints of the project, these two, otherwise extremely important biological elements, were not included.

Does 18S can´t detect Euglena and other euglenids?

The specific primers chosen to amplify the 18S rRNA gene turned out be unable to detect euglenids in water samples. Instead, we are using information from 16S "chloroplast" to detect this group.

Answer in detail:
Tests with 18S sequences in-silico PCRs (TestPrime in SILVA) showed that Excavata;Discoba;Euglenozoa... and other small related euglenoids are not covered. Instead, we are using information from 16S "chloroplast" to detect the group euglenids.

Which taxonomic level is achievable with which marker?

While the genetic markers for fish and for diatoms were highly specific, the markers for bacteria and phytoplankton were more general. Therefore, the first group of markers can achieve classifications at the species level, while the second group of markers detect mainly genera or higher taxonomic ranks. To identify organisms at species level, very specific primers in combination with specific taxonomic reference databases should be used. Possibly, further phylogenetic analyses should be performed to optimize the results.

Answer in detail: While the marker for fish (12S) and for diatom (rbcL) were highly specialized, the marker for phytoplankton (18S) and bacteria (16S) were more general. In result, the first group achieves species level, while the second group detected organisms mainly on genus or order level. To identify organisms on species level, very specific primers in combination with specific gen reference data bases must be applied to phylogenetic close related groups.

Which taxa were detected with each marker in the EAW data set?

The full list of taxa and genotypes detected using HTS within the EAW 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 EAW

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 EAW taxa analysis tool.

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

The EAW project recommends sterile encapsulated filters (Sterivex), DNA-free bottles and gloves to reduce contamination. Detailed information about sampling and precautions that must be considered are provided in our YouTube videos 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 unkown taxa were recorded by HTS?

Additional analyses, such as BLAST queries, can provide deeper understanding of closely related taxa and groups. Improved genetic reference databases, which are curated for a specific taxonomic groups 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. BLASTinduced changes in the taxon names for selected ASVs in 16S were marked in the EAW taxa analysis tool.
Answer in detail: For cyanobacteria 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. BLAST induced changes in taxon names for selected ASVs in 16S were marked in the EAW 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 EAW project to analyse fish biodiversity in alpine waters. These closed filter cartridges (VigiDNA®, Spygen®) are used to filter large volumes of water (30L) 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.

Answer in detail:
VigiDNA® is the product name of the filter cartridges (Spygen®) used in the EAW project to analyse fish biodiversity in alpine waters. The VigiDNA® sampling 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 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). Using this setup, mounted on a boat, allows collection of water along lake shore transects or in the main river current. After filtration, the filter cartridge is filled with a preservation buffer and stored at room temperature until DNA extraction. Detailed information on the sampling strategies used for fish eDNA sampling is provided at protocol section at webpage.

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

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.
Answer in detail: Basically, this system is suitable for any type of river and allows up to 30 litres of water to be filtered with a single cartridge. However, it could be challenging in low land rivers, as an increased particle load in the water can cause the filter to clog before the desired 30 litres are filtered. In all water bodies sampled with the VigiDNA system in the EAW project area, clogging due to high fine sediment loads was never an issue and 30 litres were filtered through each cartridge. When planning and conducting sampling, it is important to consider weather reports and adapt sampling to the conditions. Since all rivers are subject to increased particle transport during flood events, sampling should not take place during or shortly after such events.

Which primer pair is used for the fish eDNA analysis?

For the sequencing of fish eDNA samples, the MiFish-U primers (Miya et al. 2015) were used. This primer pair is regularly used for fish metabarcoding studies.

Answer in detail:
For the sequencing of fish eDNA samples, the MiFish-U primers (forward: 5`-3` GTCGGTAAAACTCGTGCCAGC, reverse: 5`-3` CATAGTGGGGTATCTAATCCCAGTTTG, Miya et al. 2015) were used. The mean amplicon length is 171 base pairs. This primer pair is well suited to assess the biodiversity of fishes and is regularly used for fish metabarcoding studies. However, for some genera it is not possible to distinguish between the individual species since the amplified DNA sequence is too similar, e.g. the genus Leuciscus.

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.

Answer in detail:
This depends on the sampling design. Since the eDNA is constantly transported downstream, the subsequent analysis can only detect fish species that occur upstream of the sampling site. Therefore, numerous sampling sites along the river course would be required to identify specific sections where species are present or absent. In addition, the amount of DNA released varies by species and the transport distance of eDNA in the river varies with river size and flow characteristics. In the EAW project, most of the sampling in rivers was carried out at only one site per river. This allowed us to assess the species diversity of fish upstream of the sampling sites, but with this approach it is not possible to assign specific detections to specific river sections.

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 statements on absolute abundances of different fish species based on the number of detected reads.

Answer in detail: No, so far it is not possible to make statements about the absolute abundance of different fish species based on the number of detected reads. Several studies have shown that it is possible to make accurate abundance estimates based on sequence counts, but this only worked in controlled environments (e.g. laboratory fish tanks or small ponds). However, in natural systems many factors can influence our results and may lead to incorrect assumptions. I would like to illustrate this with 2 examples: First, fish of different sizes of the same species release different amounts of DNA to their environment, which makes it difficult to estimate the number of individuals. Second, fish carcasses release more DNA than live fish, so it is possible to get very high read counts even though this species is not as common as the number of detected sequences would suggest.

Which different approaches for fish eDNA assessment were used in the EAW project?

In total, 3 different approaches were used. The VigiDNA approach, where 30 litres of water are filtered through an enclosed filter cartridge. The Sterivex® 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) point sampling approach, where 5 litres of water were collected at traditional sampling sites.

Answer in detail: A total of 3 different approaches were used. All project partners in the EAW project carried out the VigiDNA® approach, where 30 litres of water are filtered through an enclosed filter cartridge (0.45 µm). France and Italy additionally carried out the Sterivex® point sampling approach, where 2 litres of water are collected at the beginning, middle and end of each VigiDNA® transect at the lakeshore and filtered through enclosed filter cartridges (0.45 µm). In Germany and Austria, the GFC (glass fibre filter) approach was additionally used, where 5 litres of water were collected at traditional sampling sites (gillnet sites and electrofishing sites) and filtered through GFC filters (1.2 µm) using open filtration.

Join our EAW Alpine Network at:
https://www.alpine-space.org/projects/eco-alpswater/en/project-results/eaw-alpine-network
and follow our EAW activities further!

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Annex I - Recommendation flyer (on 2 pages)

Recommendations

Recommendations for the inclusion of the innovative monitoring approach in water quality assessment and management with focus on EU Water Framework directive and Switzerland WPO

Interreg Alpine Space Eco-AlpsWater

The Eco-AlpsWater (EAW) results contribute to a decisive improvement in future monitoring of biological quality assessment. Traditional monitoring methods have many known limitations, such as: difficulties in determination of indicator taxa, difficulties in finding hidden and rare taxa, selectivity of traditional methods regarding certain taxa and low number of qualified taxonomists.

The EAW methods are already able to overcome some of these limitations and offer the possibility to answer additional questions. Especially the taxa determination as the main normative element of the required ”composition“ is supported and improved considerably using the HTS methods.

Stakeholders are interested in a rating of the applicability of metabarcoding approach in terms of cost, practical handling and processing. What have we learned about it?

We can conclude that the cost for the sampling and analysing of one environmental sample was less than for the traditional analysis by light microscopy or by fisheries.

An important project outcome is that sampling protocols have been harmonised, tested and improved and are now publicly available (see project website). The biofilm protocols became even technical reports to CEN.

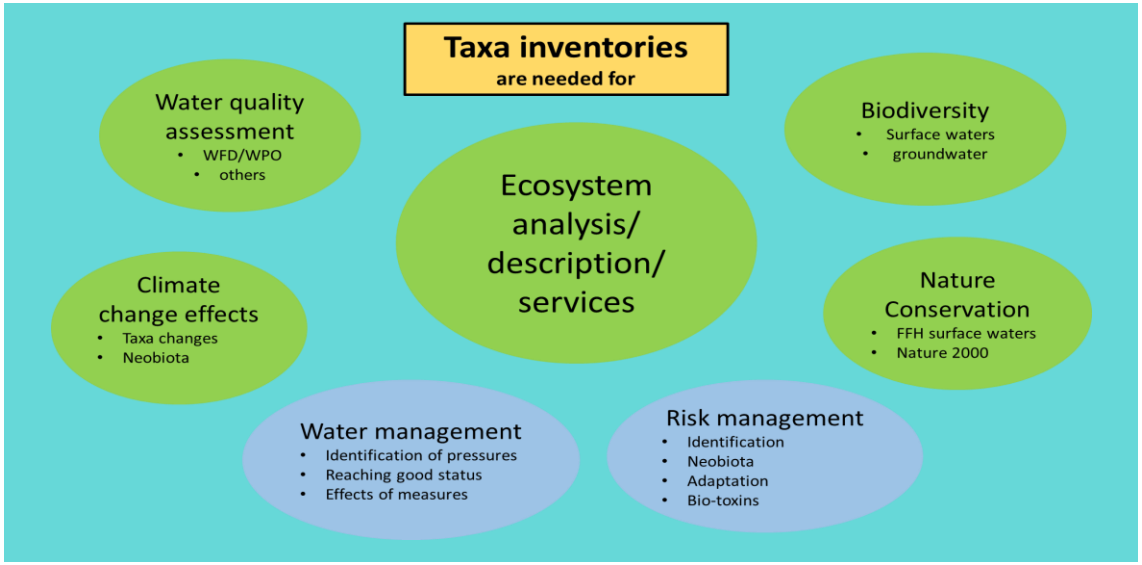
These sampling and DNA extraction protocols turned out to be easy to handle and are no obstacle for routine monitoring. The protocols were successfully applied not only at the pilot sites but also at many additional sites. Alpine freshwaters frequently show an extremely low plankton content, but the sterile filters proved to be very sensitive in terms of their taxa composition.. It is also remarkable that no contamination and no cross contamination in the EAW sample sets were detected, and all blind samples were blank (signals below detection criteria).

In case of sampling eDNA of fishes, the newly applied “VigiDNA system” is very time effective. However, the buying process of suitable large volume filters and its storage until sequencing is still of special vulnerability for DNA degradation and have to be optimized.

Interreg Alpine Space Eco-AlpsWater

To fulfil the requirements of the WFD/WPO a biological monitoring is based on the assessment of taxa inventories in freshwaters. They are not only elementary in WFD/WPO but are also of great use for other topics in ecosystem analysis and water management

Figure on right: Illustration of public interests in inland waters to which taxa inventories are relevant.



EAW metabarcoding methods can fill the WFD/WPO gaps in water quality assessment and management:

The EU Member States are dealing very differently with some elements, not covering all required groups (e.g. focus on filamentous green algae or only diatoms). >>> An homogenous EAW approach for pigmented microbial taxa living in biofilms of freshwater substrata named "phytobenthos" becomes possible with the multi-gen-marker approach.

Fish monitoring, especially in lakes, is very time consuming and expensive. >>> The new EAW HTS approach is non-invasive and allows fish species to be detected without harming them, is much more sensitive and cheaper.

HTS metabarcoding already detect microzoobenthos/-plankton by 18S and bacteria by 16S in the plankton and in biofilms. >>> There is a chance to improve this HTS method for pelagic zooplankton and bacteria taxa determination.

The use of biological quality elements in the WFD is required in a different way for the different kinds of monitoring. For investigative monitoring no requirements are given to identify unknown pressures. >>> Here, the application of the EAW eDNA metabarcoding has the highest prospect to support or even monitor it completely in an effective and efficient way. Additionally, it can also be integrated for surveillance and operative monitoring programs.

The methods of the WFD are often not suited to detect minor changes in the ecosystem in response to measures and lack sensitive and easy-to-use methods. >>> Water management could be improved by molecular–based tools and by metrics detecting the biological effect of measures applied on freshwaters, which are failing the good ecological status.

The identification of neobiota at an early stage, with initially low population levels or hidden status, could be supported by EAW methods due to their higher sensitivity, as well as the identification of the presence of toxic or hazardousspecies like some cyanobacteria (blue-green algae). The EAW methods could support the implementation of the swimming water directive. Identification of invasive and/or potentially harmful species in an early state may improve risk management policies and improve drinking water protection in reservoirs.