Eco-AlpsWater

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

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

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Recommendations for the inclusion of innovative monitoring approaches in water quality assessment and management (WFD/WPO)

Introduction

This is an online report with recommendations for the inclusion of innovative monitoring complementing traditional approaches used in water quality assessment and management (WFD/WPO) of Alpine blue infrastructures. This report reflects also the feedback from regional meetings with observers and regional stakeholders (Activity A.T4.3.). The aim was to identify the criteria for implement new monitoring approaches in the assess of ecological status of water bodies (WFD/WPO) in the 6 countries. In meetings organized by governments in collaboration with project partners, the concrete inclusion of new approaches in other hydrographic basins/regions was recommended and planed. Additionally, activities were in compliance with strategies developed at a wider AS/EU level (e.g. EUSALP AG6, DNAqua-Net guidelines etc.). In this part, the following issues are highlighted:

At first, we provide a broad overview of the requirements of the WFD/WPO, which is essential to evaluate the potential contribution of the metabarcoding approach to the water quality assessment of freshwaters.

In the next step we highlight the connection of the WFD/WPO to further topics of ecosystem analyse and water management.

During meetings, we also identified the stakeholder interest for rating the applicability of metabarcoding approach in terms of cost, practical handling and processing (Field of interests see delivery D T.4.1.1).

Finally, we evaluate the possibilities of implementation of the EAW innovative monitoring approaches in water quality assessment and management.

1. Requirements of the WFD

The EU's Water Framework Directive 2000/60/EC (WFD) aims to protect and improve inland surface waters, transitional waters, coastal waters and groundwater in order to preserve ecosystems and ensure human water use for the future. In the Alpine region, only inland surface waters such as lakes, rivers and groundwater are relevant water categories.

Some important targets of the WFD are:

- Water management on the scale of river basins (and sub basins)
- Accomplish and/or preserve at least the good ecological status or good ecological potential of surface waters.
- Accomplish and/or preserve a good status of groundwater quality
- Accomplish and/or preserve a good status of groundwater quantity to save and improve groundwater dependent ecosystems
- To eliminate hazardous substances from water ecosystems
- Offer to improve and ban of degradation of waters
- If the good status is missed, measures have to be planned and implemented

The WFD includes several requirements about

- Definition of waterbodies and waterbody types
- Determination and assessment of ecological and chemical status
- Biological and chemical quality elements
- Supporting elements e.g. chemical, physical and hydromorphological quality elements
- Content and cycle of monitoring programs
- Assessment methods and intercalibration

Some main issues concerning ecological status are listed here:

- Assessment of ecological status hast to be reported in five classes
    - High – reference conditions very close to natural conditions

    - Good – only minor degradation from high status
    - Moderate – clear degradation
    - Poor – strong degradation
    - Bad – heavy degradation far from natural conditions
- Four biological elements have to be used for determination of ecological status of surface waters
    - Phytoplankton
    - Macrophytes and Phytobenthos (Benthic flora)
    - Macroinvertebrates
    - Fish
- Additional supporting chemical, physical and hydromorphological quality elements, which describe the environmental conditions for the biological elements, have to be monitored for the ecological status assessment
- Requirement of different kinds of monitoring
    - Surveillance monitoring
    - Operational monitoring
    - Investigative monitoring
- Requirements for the biological elements in

  Rivers
    - Composition, abundance and biomass of phytoplankton (see very large rivers)
    - Composition and abundance of aquatic flora ((phytobenthos & macrophytes)
    - Composition and abundance of benthic invertebrate fauna
    - Composition, abundance and age structure of fish fauna

  Lakes
    - Composition, abundance and biomass of phytoplankton
    - Composition and abundance of other aquatic flora (phytobenthos & macrophytes)
    - Composition and abundance of benthic invertebrate fauna
    - Composition, abundance and age structure of fish fauna

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 waterbodies 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 waterbodies in high, good or worse status and which or how many measures are planned. On the other side, the methodology 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 (see chapter 5b). All of these

assessments require taxa inventory lists, which have to be compiled by specific collection and detection methods, as so the metabarcoding approach by the project Eco-AlpsWater.

2. Requirements of the WPO

The Modular Stepwise Procedure for the analysis and assessment of watercourses includes survey methods in the areas of hydrodynamics and morphology, biology, and chemical or toxic effects. The status classes developed within the Swiss Modular Stepwise Procedure are comparable with the system of ecological classes defined in the WFD (from bad to high). According to the Water Protection Ordinance (WPO 1998), methods for the determination of the ecological status on the basis of biological quality elements have been designed by the Federal Office for the Environment (FOEN). However, these protocols represent guidelines for Cantons, which have the legislative power to decide about the terms of their application in their own territory. At the moment, only reports for assessing the status of rivers and streams using diatoms and fish have been published. In 2013, FOEN published a report describing the concept for the ecological assessment of the condition of Switzerland's lakes, as an integral part of the modular stepwise procedure. It describes the use of instruments supporting the decision-making process in the development and application of modules for assessing the condition of lakes, and provides an overview of the priorities in the area of module development. Considering a scale of importance from 1 (low) to 3 (high), the development of the module plankton and fish was awarded a degree of 3 and 2, respectively. Till now, each Canton has applied internal protocols for the assessment of phytoplankton and the standardization at Federal level is under development. Moreover, the transboundary waters (Switzerland-EU country) are under the control of International commissions (i.e. CIPAIS, CIPEL, IGKB) which have specific goals and involve different types of quality indicators, which sometimes mediate between WPO and WFD requirements.

3. Connection of the WFD/WPO to further topics of ecosystem analysis and management

The most important key element to describe ecosystems and assess ecological status is the determination of taxa of the required biological quality elements (defined groups of organisms). These elements in the WFD/WPO were chosen well and they already reflect important parts of water ecosystems and their food chains. Therefore, there is a strong connection to other important fields of water environmental analyses and descriptions, water environmental protection, assessment of effects of any pressures or changes on the water environments or risks for the use of waters or water management.

Some main links of water ecosystem assessment done for implementation of the WFD/WPO are listed here:

- Water quality assessment (use for WFD/WPO, trophic state, saprobic state, acidification, salinisation, others)
- Biodiversity determination (in surface waters
- Nature Conservation (use for FFH, Natura 2000 assessment, others)
- Climate change effects (on water environments, on water use e.g. drinking water, touristic uses)
- Water management (targeting good ecological status, analysing effects of measures, others)
- Risk management (identifying neobiota and their effects on environments, support of swimming water directive, suggesting measures of adaptation for water uses, others)

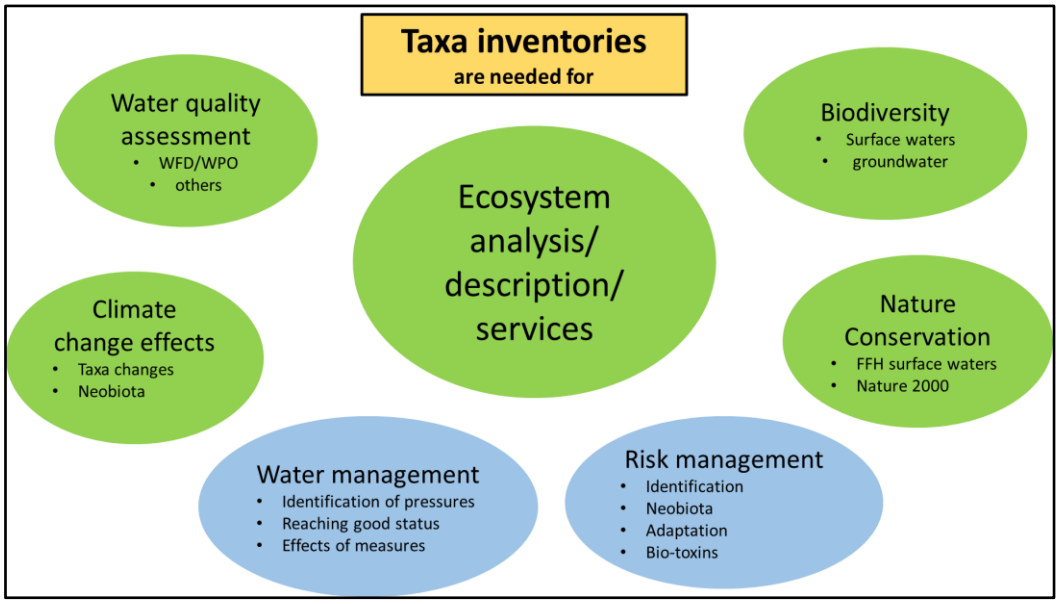

Figure 1: Illustration of public interests in inland waters to which taxa inventories are relevant.

4. Rating the applicability of metabarcoding approach in terms of cost, practical handling and processing

Costs per eDNA sample

Overall, the cost for the sampling and analysing of one environmental sample was much less than for the traditional analysis by light microscopy or by fisheries.

Costs per sample varied for gen marker, sampling material (e.g. sterile filter cartridges, Falcon tubes), DNA extraction kit, PCR amplification, and cost for the use of infrastructure such as the next generation sequencing (NGS) platform and for the bioinformatic treatments to obtain taxonomic inventories.

In the workflow of the Eco-AlpsWater project all these steps were organized by the management team specific for each bio-component and done in a directed sequence of project partners.

As a speciality of the research project, in the EAW workflow (delivery D T.1.1.2 Workflow), external services were not involved, because the project steering group decided to switch to facilities provided by partners within the project consortium to improve comparability of the results.

Excluding costs for working hours, the following costs were applied for budget calculations (costs refer to those established in 2018/2019, for a target sequencing depth of approximately 50,000):

- Cost per plankton sample: filter cartridges & DNA extraction & sequencing 16S, 18S: 115 Euro
- Cost per biofilm sample: Falcon tube & DNA extraction & sequencing rbcl, 16S, 18S: 115 Euro
- Cost per Vigi fish sample: filter cartridges & DNA extraction & sequencing 12S: 265 Euro

Additional smaller costs arise for backup-samples, sterile or sterilisation material and for sample shipping.

Estimations of costs for a future monitoring, which will be presumably based mainly on external services and infrastructures, could be higher than it was for the project partners.

Practical handling and processing of eDNA sampling and sequencing

Overall, the standardisation of handling and processing including library preparation was one of the main scopes of the Eco-AlpsWater project. It is a strong highlight that sampling protocols were harmonized, tested, improved and become public available (see project website). The biofilm protocols became even technical reports to CEN[1].

Despite the required improvement of the fish protocol, all other sampling and DNA extraction protocols are easy to handle and are no obstacle for routine monitoring. Because of this, the harmonized sampling was successfully applied not only at the pilot sites, but at many additional sites. Plankton sampling with sterile one-time Sterivex filters turned out to be high sensitive for Alpine freshwaters even with extreme low plankton content.

In case of sampling eDNA for fish detection, the newly applied "VigiDNA system" is very time saving. The sampling system developed by the company Spygen uses sterile one-time cartridges for filtering large water samples with a pumping system. Unfortunately, Spygen offers actually the proposed and applied system only in combination with other services. This type of filter cartridges are only sold in combination with company service for DNA extraction and analysis. Furthermore, the filter storage until sequencing is still of special vulnerability for DNA degradation and have to be optimized. Despite these issues, the use of large volume filters coupled with suitable eDNA preservatives remains a valuable options to assure the recovery of even small quantity of organic matter released from fish. Suitable alternatives are still under testing within the EAW project.

It is also remarkable that no contamination and no cross contamination in the EAW sample sets was detected, and all blind samples were blank (signals below detection criteria).

5. Possibilities of implementation of the EAW innovative monitoring approaches in water quality assessment and management

a) Elements of EAW project

The EAW innovative monitoring approaches cover the following biological groups, which partly cover the WFD/WPO target taxa:

- Phytoplankton
- Biofilms incl. Benthic diatoms, Cyanobacteria, Cyanotoxins, Ciliates, Bacteria
- Fish

For these biological groups the following products were developed in the EAW project:

- Protocols for sampling procedures and eDNA-extraction and analyses (WP1)
- Protocols for bioinformatic treatments of next generation sequencing (NGS) data (WP1)
- Implementation of the metabarcoding approach based on protocols and workflows at pilot sites and additional lakes and rivers in 6 countries.
- Results of comparisons of eDNA sampling and treatments with classical biological monitoring methods like direct sampling or detecting of water organisms, taxa

[1] CEN , 2018a. Water quality CEN/TR 17244 Technical report for the management of diatom barcodes 1 11.

CEN , 2018b. Water quality CEN/TR 17245 Technical report for the routine sampling of benthic diatoms from rivers and lakes adapted for metabarcoding analyses. CEN standard 18.

determination, counting, abundance or biomass determination from alpine rivers and lakes.
- HTS metabarcoding detection of additional organism groups: e.g. microzoobenthos/-plankton by 18S and heterotrophic and cyanobacteria by 16S in plankton and biofilm samples (e.g. list of ciliate taxa in Annex 2 delivery D T.4.1.1).
- Project storage database containing all biological and environmental data including sequencing files from all samples of the project waters.
- Database for the practical use in the participating countries containing all organism data comparable by a common taxa ID (EAW taxa analysis tool).

A detailed and complete description of this Eco-AlpsWater tool-box for the implementation of the applied innovative monitoring approaches is available in the public deliverable D.T3.5.1.

b) **Direct links to the WFD/WPO**

The EAW project results can directly be connected to the WFD/WPO required biological quality elements

- Phytoplankton
- Phytobenthos as part of aquatic flora
- Fish

Deliveries of WP2 demonstrated that the biological metrics used in the traditional methods differ between each biological quality element, waterbody category (lake or river) and country. The so-called metrics with indicator lists are far from being equal in the Alpine Space, but at least the assessment results were equalized in a specific WFD intercalibration process (see Commission Decision (EU) 2008, 2013, 2018).

The following table provides a list of all microbial metrics, bases of taxa inventories and indicator lists used in the countries participating in the Eco-AlpsWater project.

Concerning fish community assessment, there are national WFD/WTO methods for rivers in each country, but for lakes only few have WFD/WTO-compliant monitoring methods.

Full descriptions of national biological quality element monitoring methods are available in Eco-AlpsWater Deliverable D.T2.1.1 titled “Mutual awareness, learning and exchange of experiences and approaches in water quality assessment among PPs (public)”.

The results of the EAW project contribute to a decisive improvement in future monitoring of biological quality elements. Traditional monitoring methods have many known limitations, the proposed EAW metabarcoding approach can be used to complement traditional methods and overcome these limitations. These limitations include

- Difficulties in separation and determination of important indicator taxa.
- Difficulties in finding hidden/rare taxa by the classical sampling methods.
- Selectivity of traditional methods regarding certain taxa.
- Number of taxonomists is decreasing due to scientific focus on molecular methods.

The EAW methods are already able to overcome some of these limitations and offer the possibility to answer additional, previously unaddressed, questions after the data set has been improved and completed. Especially the taxa determination as main element of the requirement „composition“ could be supported and improved considerably using the EAW HTS methods. Actually, the DNA metabarcoding of benthic diatom is very promising (see Deliverable D.T1.3.3; Pérez-Burillo et al. 2020).

Table 1: List of metrics and method names used for the assessment of water quality sorted for participating countries and bio-components

country	WB category	Bio-component	index name for assessment	method name assessment
IT	lakes	biofilm diatoms	EPI-L_index	EPI-L (Report CNR-ISE, 02.13_2018)
IT	river	biofilm diatoms	ICMI Index	INTERCALIBRATION COMMON METRIC INDEX - ISS Rapporti ISTISAN 09/2019
IT	lakes	plankton	PTIot	PTIot (Report CNR-ISE, 02.13_2018)
AT	lake	biofilm diatoms	No national method	No national method
AT	river	biofilm diatoms	Phytobenthos-Gesamtbewertung	Ecoprof (aktuelle Version: 5.0.4)
AT	lakes	plankton	EQR of Modul Brettum-Index	EQR_Euphot-ver.2013
FR	lake	biofilm diatoms	Method under development	
FR	river	biofilm diatoms	Biological Diatom Index (BDI)	DBI 2009 Coste et al. 2009
FR	lakes	plankton	Phytoplankton Index for Lakes (IPLAC)	Laplace-Treyture & Feret, 2016.
DE	lake	biofilm diatoms	Diatomeen-Index Seen	PHYLIB 5.3.0 (18.02.2016)
DE	river	biofilm diatoms	Diatomeenindex Fließgewässer (DIFG) – applied in project test	PHYLIB 5.3.0 (18.02.2016) (3 modules: DIFG, phytobenthos without diatoms and macrophytes)
DE	lakes	plankton	Phyto-See-Index	PhytoSee 7.0 (15.12.2017)
CH	lake	biofilm diatoms	No national method	No national method
CH	river	biofilm diatoms	Swiss Diatom Index (DI-CH)	OFEV, 2007. Méthodes d'analyse et d'appréciation des cours d'eau Diatomées - Niveau R (region)
CH	lakes	plankton	National Method under development (method available at Canton level)	OFEV, 2013. Système d'analyse et d'appréciation des lacs en Suisse
SI	lake	biofilm diatoms	Rott's trophic index	Slovenian national method (using Rott's trophic index)
SI	river	biofilm diatoms	Rott's trophic and saprobic index	Slovenian national method (using Rott's trophic and saprobic index)
SI	lakes	plankton	EQR of Modul Brettum-Index	EQR_Euphot-ver.2013

For the WFD assessment of surface waters, taxa inventories of all four biological quality elements are needed in the worst case of surveillance monitoring. Actually, the EAW results do not cover the biological quality-elements macrophytes and macroinvertebrates. Therefore, a complete WFD assessment is not possible solely based on the eDNA data of the project. However, there are other projects running on developing DNA – methods for macroinvertebrates for example with marker gene CO1 (Elbrecht & Leese, 2017, Elbrecht et al. 2017, see also DNAqua project.). Similarly, macrophytes detection by eDNA is under development (Anglès d'Auriac et al. 2019). If these approaches achieve good recovery rates and completeness of taxa inventories, DNA/eDNA methods will cover almost the complete set of biological quality-elements required by the WFD (Greyer et al. 2018) in near future.

Although the eDNA metabarcoding approach is well suited to fulfil many of the requirements for WFD-compliant assessments, there are questions that cannot be answered by using this methodology, such as age distribution, biomass or total abundances. Attempts to handle quantification aspects are promising (Vasselon et al. 2018).

c) Gaps of the WFD/WPO possibly to be filled by EWA methods

- The EU Member States are dealing very differently with the implementation of the biological quality element "phytobenthos" (table 1). In some countries, there are only some filamentous green algae included in the element "macrophytes", in other countries only diatoms are used, excluding other algae. Complete determinations of microalgae and cyanobacteria living in the biofilm are performed only in Austrian and German rivers. Besides diatoms, in Slovenia other organisms are only examined with relative presence (classes 1-5), whereas only diatoms are used for calculations of indexes. Therefore, the use of phytobenthos is quite heterogeneous across Europe. With the help of the EAW metabarcoding approach using several gene markers, there would be a chance for the development of a more homogenous approach for phytobenthos, covering all important assessment aspects of this quality element.

- Fish monitoring, especially in lakes, is very time consuming and expensive. In addition, gill nets are used for the traditional fish stock assessment, which lead to the death of the animals and thus to conflicts with the Animal Welfare Act. The new HTS approaches are non-invasive, allow detection of fish species without harming them and are more sensitive and cheaper. Zooplankton, bacteria and fungi are important parts in the food chain of lakes and partly in rivers, but these organisms are not required in the WFD monitoring. Zooplankton occurs as consumer of the primary production and as food-source for higher consumers like fish in lakes. Bacteria are an important food-source for zooplankton and macroinvertebrates. 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, by using more specific markers, other metazoans and fungi.

- The use of biological quality elements in the WFD is required in a different way for the different kinds of monitoring. For surveillance monitoring all four elements have to be used, for operational monitoring only the elements which are dependent on the main pressures should be used. For investigative monitoring no requirements are given. Investigative monitoring has to be carried out in order to identify unknown pressures. EAW Methods could help to support surveillance monitoring, and in future probably replace operative monitoring in special cases e.g. trophic pressures. Investigative monitoring could be supported by eDNA metabarcoding or maybe done completely in an effective and efficient way.

d) **Additional Topics to be covered by EWA methods** (listed under 3.)

- Water quality assessment additional to WFD/WPO requirements.

  Trophic state, saprobic incl. microsaprobic state, state of acidification, salinization or pressures caused by hydromorphology degradation.

- Biodiversity determination- To assess changes in biodiversity, the determination of a preferably complete inventory of the species composition in an ecosystem is necessary. The traditional methods, used for biodiversity assessment, are not able to fulfil this requirement. Here, the HTS methods can be of major importance to complete the required knowledge. Up to now, there is little knowledge about the biodiversity in groundwater ecosystems. The use of EAW methods could be an important tool, to close knowledge gaps regarding the species compositions of these habitats.

- Assessment for nature conservation purposes could be supported with EAW-Methods. The monitoring approaches for the FFH/Nature 2000 directives are quite less strict then those of the WFD. Almost no requirements are given to asses water ecosystems for implementation of nature protection directives.

- To assess climate change effects on water environments especially on water quality, there is no legislation so far. Partly the WFD/WPO methods could be used for such a monitoring but they are not sufficient to cover all relevant questions occurring. In addition, qualitative effects on groundwater might be important for assessing changes, which could be of importance for future management of groundwater resources. With the EAW methods, the development of necessary assessment tools could be supported.

- Water management could be improved or supported by EAW HTS methods. Especially for the detection of measure effects, tools or metrics could be developed. In many cases, easy-to-use methods that show the direction of a system's development are needed as a first step in assessing the responses of aquatic ecosystems to measures. The methods of the Water Framework Directive are often not sensitive enough to detect minor changes in the ecosystem. Therefore, helpful methods to support risk management of water ecosystems are needed (Darling & Mahon 2011). The identification of neobiota in early introduction states, with low abundances 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 dangerous species like some blue-green cyanobacteria. The EAW methods could support the implementation of the swimming water directive. Identification of invasive and/or potentially dangerous species in an early state may improve risk management policies and improve drinking water protection in reservoirs.

e) Conclusions about the implementation of the EAW innovative monitoring approaches

The recently used methods for implementation the WFD/WPO, especially for the assessment of ecological status, are based on taxa determination using traditional methods like light-microscopy. These methods are quite useful and can achieve results of high quality, but the quality depends strongly on the knowledge of the biologist and the quality of available determination literature. For each biological quality element, biological specialists with good experience are necessary to achieve meaningful results. Specialists are needed to develop, implement and integrate the novel and traditional methods into the next generation water biomonitoring systems. The following table summarizes advantages and challenges of both, traditional and molecular methods.

Table 2: Comparison of traditional (WFD/WPO) and new methodology of taxonomic identification

Criteria	Traditional method	New EAW eDNA method
Output	Taxa inventories	Taxa lists in a much wider spectrum, depending on gene marker regions.
Taxonomic resolution level	Genus/(sub-)species sometimes lower	Genus/species or higher taxonomic ranks, depending on gene marker regions
Comparability	Lower: depends on persons knowledge	High and objective
Equipment	Easy	Advanced; costly infrastructure
Staff	Biologists/specialists	Lab. people, IT- and storage facilities, biologists
Effort	Time consuming and specialized	Quick but specialized; automation for processing will reduce time
Selectivity	Methods can be selective regarding certain species	Methods are non-selective
Sensitivity	Low abundant organisms are hard to detect	Methods are sensitive enough to detect low abundant and/or elusive species

Right now, we assume that a combination of both methods should be the first step to introduce the new EAW approaches into the applied assessment methods. For a fast improvement of methods and output such a combination of methods brings people of both disciplines together, which seems to be an exquisite way to further develop the scientific discussion and method development. More projects like the Interreg EAW project are required to bring forward the implementation of the eDNA metabarcoding into regular monitoring.

f) **Future prospects for implementation of the EAW innovative monitoring approaches**

eDNA metabarcoding is revealing one of the tools of choice of the 21st century for fundamental research and the future of large-scale biodiversity monitoring programs, thanks to its cost-effectiveness and relatively easy implementation. Expecting such a broad application of eDNA methods, this also offers the opportunities and new perspectives within the WFD/WPO:

- Possibility for the use of additional primers for a better coverage of different biological quality elements and species or increase the depth of determination.
- Combination of traditional and eDNA approaches allows biodiversity assessment at an unprecedented level.
- Cost efficient eDNA approaches are perfectly suited for large-scale, continuous 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.
- Interdisciplinary collaborations are needed for further development or improvement of assessment methods based on eDNA/DNA.
- Additional projects to further improve molecular EAW methods would ensure the establishment of these methods and create a standard for ecological monitoring purposes.

6. Literature:

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Commission Decision 2013/480/EU of 20 September 2013 establishing, pursuant to Directive 2000/60/EC of the European Parliament and of the Council, the values of the Member State monitoring system classifications as a result of the intercalibration exercise and repealing Decision 2008/915/EC (OJ L 266, 8.10.2013).

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