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Alpine waters & eDNA

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

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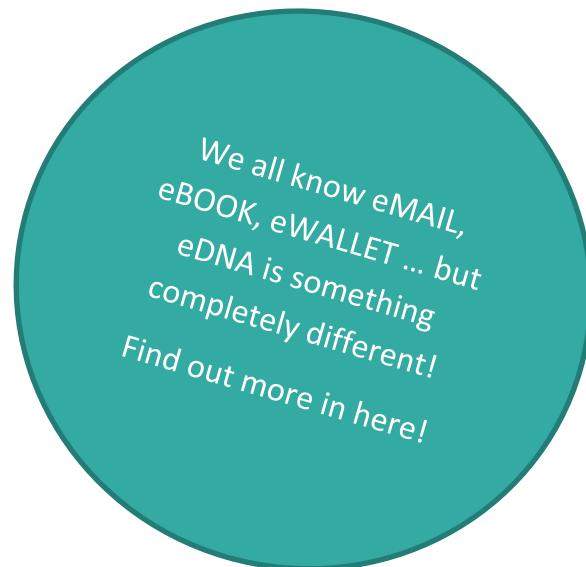
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All actions in the environment are reflected in the health of our waters.



Introduction and EAW pilot areas

Inland waters account for only a few percent of the world's water, but their role on our planet is invaluable. These waters are one of the most precious natural resources on Earth, and at the same time they are highly endangered due to human activities. Many surface water bodies are affected by pollution and degradation of ecosystem functionality. Quality and clean water resources are primarily important for the provision of drinking water, but they are also crucial for hygiene and health care, agriculture, industry, recreation, tourism and other ecosystem services. The basis for effective management of water resources is the regular monitoring of water status, which allows us to detect changes in the environment. By monitoring taxa inventories and various biological indicators we can recognise a deterioration of the situation in time and take appropriate action.

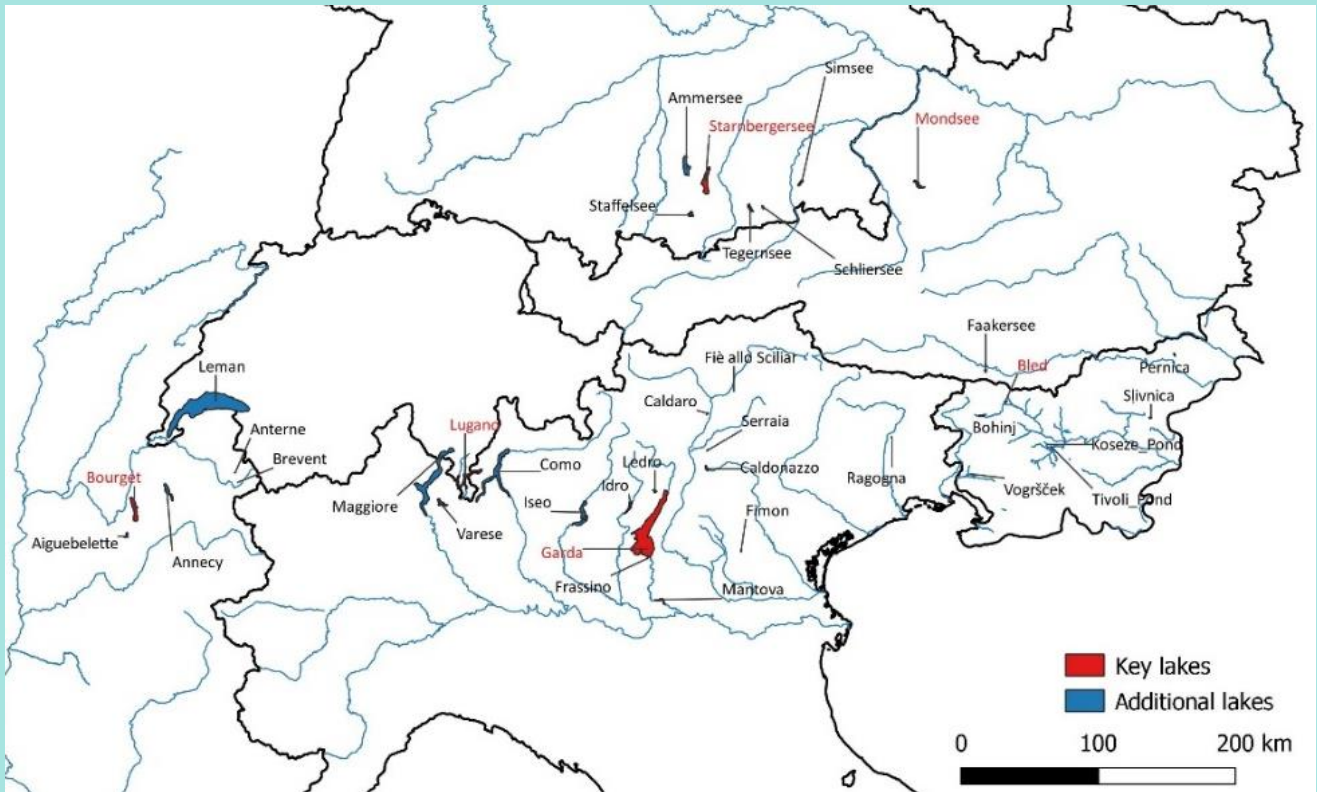
The Eco-AlpsWater (EAW) website offers a lot of pictorial and video material:

<https://www.alpine-space.org/eco-alpswater>

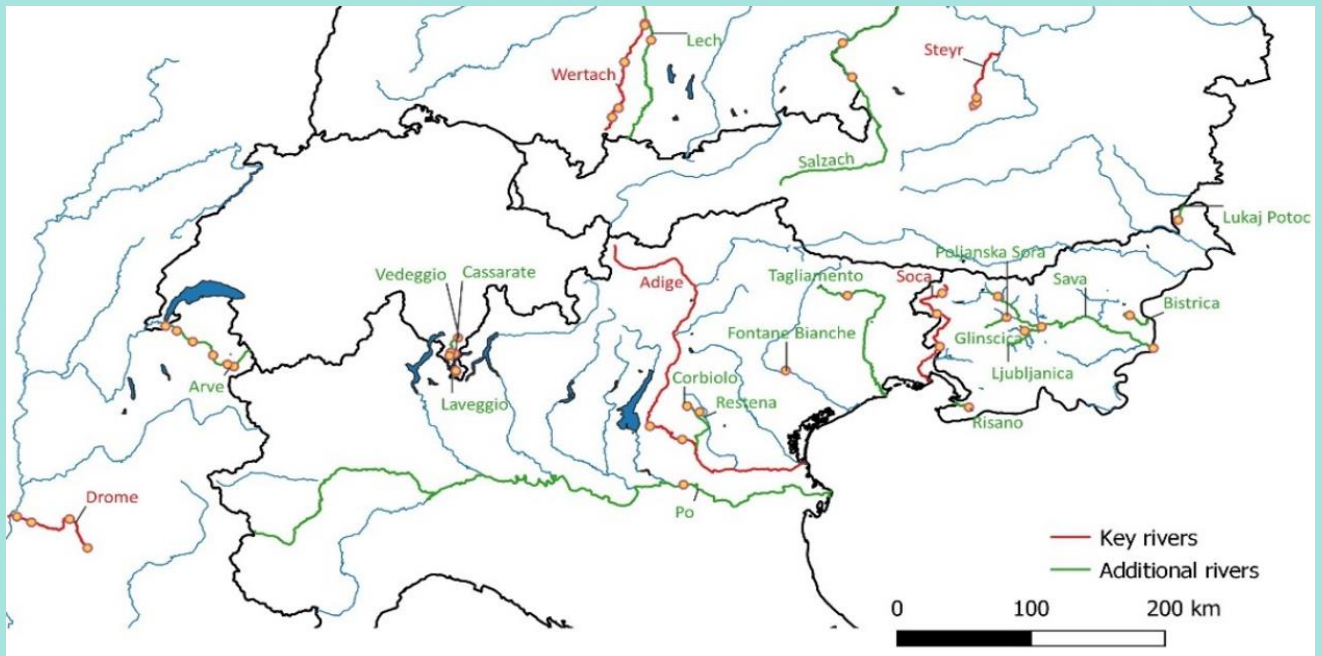
Short project video:

<https://www.youtube.com/watch?v=VIKk9LVRqho>

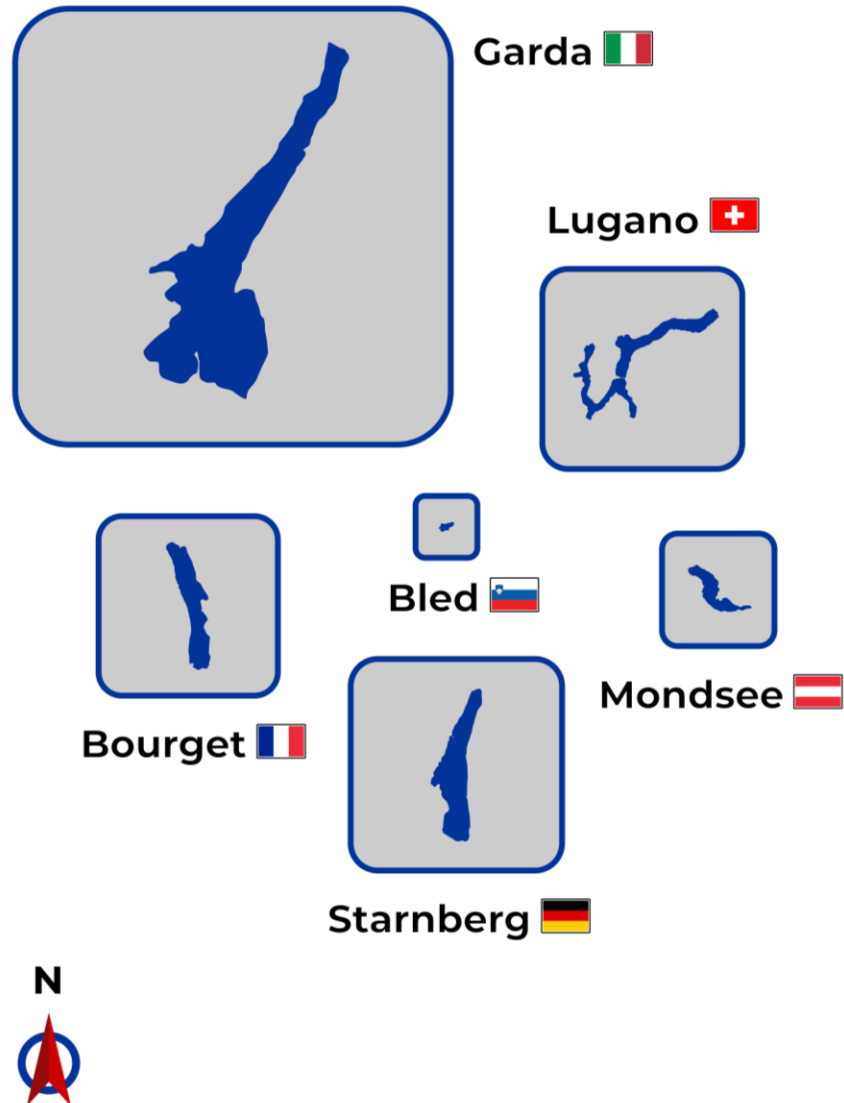
Monitoring the ecological status of waters can be done with traditional and/or modern methods. However, new methods must first be developed, optimized and a thorough comparison regarding the results of both approaches must be conducted. This is exactly what the [Eco-AlpsWater](#) (EAW) project is about, where a consortium of 12 partners from Alpine countries took care of the latest scientific approaches. They tested them in pilot areas and then linked this new findings and experiences with decision makers and water managers. The acronym of the EAW project represents the Innovative Ecological Assessment and the Water Management Strategy for the Protection of Ecosystem Services in Alpine Lakes and Rivers. The main goal of this European project is to improve traditional approaches to monitoring the ecological status of waters by using advanced next-generation DNA sequencing techniques (metabarcoding). The new approach uses high throughput sequencing analysis to analyze the DNA included in microscopic bacteria, algae and other microorganisms, or released by larger aquatic plants and animals, including fish (environmental DNA, eDNA). The novel techniques allow rapid and cost-effective identification of species and smart data processing, data storage and information retrieval of big data).



EAW key lakes: Bled, Bourget, Garda, Lugano, Mondsee and Starnberg.



EAW key rivers: Adige, Drome, Soča, Steyr and Wertach.



The key lakes in Austria, France, Italy, Germany, Slovenia and Switzerland were sampled at monthly interval, obtaining a final set of over 180 samples. Those lakes are very diverse regarding size and biochemical characteristics. Therefore, the new molecular methods were also tested on 150 samples from additional water bodies in the Alpine regions of previously mentioned countries, for a total of over 330 samples analysed.



Human impact on inland water ecosystems

Humans, have a significant impact on water quality, natural processes, and the structure and shape of running and standing waters through their way of life and their interferences in the environment. Human impact can change the aquatic environment to such an extent that existing organisms are no longer able to adapt to the new conditions. This process leads to a change in the natural species composition, native species become fewer or disappear altogether, and new, potentially invasive species fill these gaps with potentially negative consequences for the ecosystem and associated ecosystem services.

An **ecosystem** consists of organisms and the physical environment with which these organisms are associated. The **aquatic ecosystem** includes organisms that live in or on water and their environment - water, sediment, riparian zone, etc. Examples of inland water ecosystems are streams, rivers, ponds, lakes and wetlands.



Examples of human intervention in aquatic environments: intensive use, loading of nutrients and organic substances with wastewater discharges, washing of surfaces, roads and air, drainage, irrigation, construction of walls, thresholds and barriers.

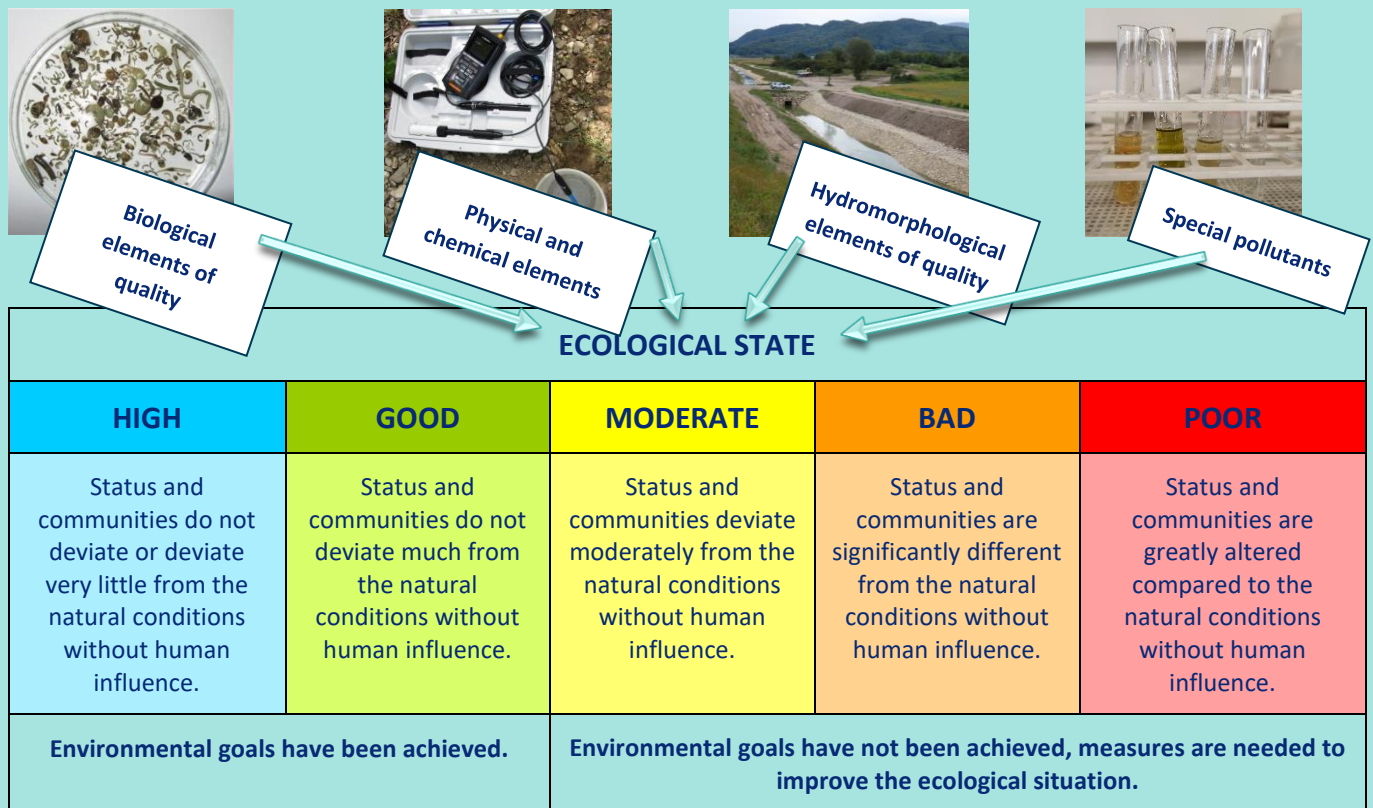
Humans have always exploited freshwater and riparian space. With the development of agriculture and urbanization, we drained numerous wetlands and flood plains, and for flood protection, we levelled riverbeds, built dams and in many places surrounded them with flood walls. With intensive use of space, we often change the characteristic riparian zones of rivers and lakes. In addition, we are changing the water chemistry, e.g. eutrophication causes algal blooms, which is the biological response to nutrient overload from agriculture and wastewater.



Conventional assessment of the ecological status

What is an ecological status?

In order to improve and/or maintain the condition of water, it is necessary to know and regularly monitor their condition. To this end, also the Alpine countries carry out water **monitoring** following regulations given on national (CH-WPO) or European level (WFD) and evaluate the **ecological status of waters**. This status tells us in what conditions aquatic ecosystems and their associated organisms like microalgae, plants and animals are. Some species are important indicators for determining the status of water bodies; therefore, a precise and objective determination is necessary. Beside the biological, also chemical and hydrological aspects are considered to assess the ecological status as accurate as possible. However, the EAW project focused only on the biological aspects. Additional monitoring measures are carried out to answer other questions, such as whether **the water is safe for swimming, suitable for drinking, watering or other usages**.

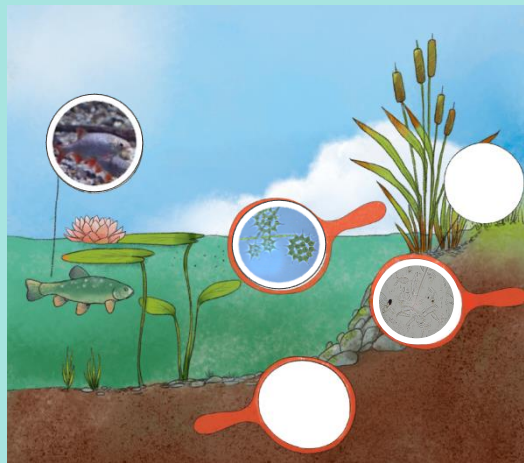


The conventional assessment of ecological status is illustrated by a five-classes color scale, and the normative description of each class is redrawn from the European Water Framework Directive (WFD).



A color scale ranging from high (blue) to poor (red) is used to illustrate the ecological status. Across Europe, all water managers have agreed on the same goal, namely to keep all water bodies in good or very good ecological status. A comparison of the actual situation with the expected natural state tells us how much deviation has occurred due to human activities.

Waters differ from each other (e.g. regarding flow characteristics, sediment transport, light exposure, depth or nutrient concentrations), and each type of water has its own characteristic plants and animals species composition. Lakes and rivers are classified into different **water body types**. For example Alpine lakes share different characters than lowland lakes, and are assessed with other indicators and nutrient boundaries.



Each biological quality element responds differently to different pressures and changes in the aquatic ecosystem, so it can serve as an indicator. In the EAW project, phytobenthos, phytoplankton and fish were selected for analysis.

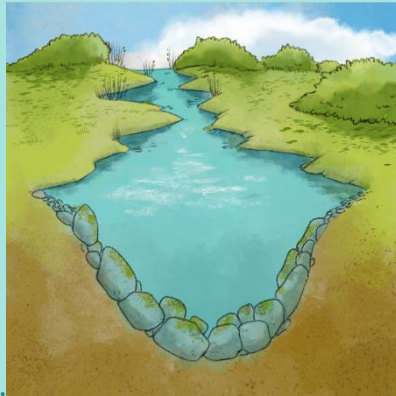
The presence or absence of certain organisms is a good indicator of the ecological status of a particular habitat; these organisms are called **indicator organisms** or **bio-indicators**. They need specific conditions for growth and reproduction and are very sensitive to changes, which means that in the case of a habitat degradation, they no longer occur or their abundance changes. To determine the status of a lake or river precisely, several biological quality elements are considered, some of which are described below.

For detailed information regarding sampling of the different biological quality elements (phytobenthos, phytoplankton and fish) please visit the EAW webpage ([documents](#)). Sampling can be seen also in different videos at EAW webpage ([videos](#)).



Phytobenthos

Phytobenthos are micro-algae (e.g. green algae, euglenophytes, red algae, yellow algae or siliceous algae called diatoms) and cyanobacteria that live attached to the substrate (stones, macrophytes, roots, wood and plant debris). In the EAW project, we focused mainly on the phytobenthic community of diatoms, which have an external cell layer made of silica.



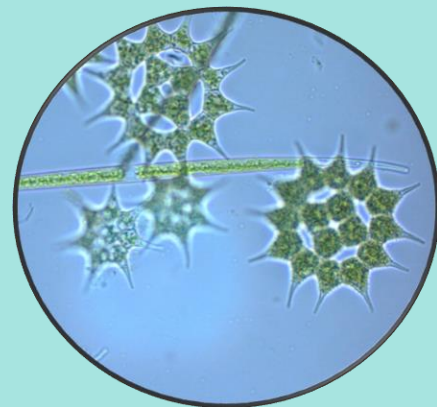
The algae biofilm on stones, collected in rivers or lakes, is scraped into a container using a brush. In the laboratory, the organic matter in the cells is removed with acid, leaving only siliceous frustules (shells). The purified sample is then loaded on a microscopic slide and analysed regarding diatom species presences and abundances, using a light microscope.

Different diatom species are distinguished by the shape and structure of the frustules. Due to the general prevalence of diatoms and their rapid response to changes in the environment, they are a good indicator of water quality. Based on the indicator species found in the sample and their frequency, we calculate the trophic and saprobic index and the ecological status for nutrient (trophic) and organic (saprobic) load.



Phytoplankton

Phytoplankton is a large and very species-rich group of floating micro-algae and cyanobacteria. All of them share the green pigment chlorophyll a for primary production, which can be used as a proxy for their biomass. Although some of them can swim using flagella or regulate their position in a specific water depth by gas vesicles, they are mainly transported and drifted by the currents of the water masses. Therefore, phytoplankton is important especially in slow-flowing and stagnant waters and can cause algal blooms with toxins. Before sampling, a special disk is used to determine the depth at which there is still enough light to thrive and thus the depth up to which samples are taken (starting at the water surface). The traditional method is very time consuming and biologist have to know many morphological traits for species determination. Supportive, plankton-net samples contain a concentrate of living cells, but they are not suitable for counting, because the net is selective regarding larger cells.



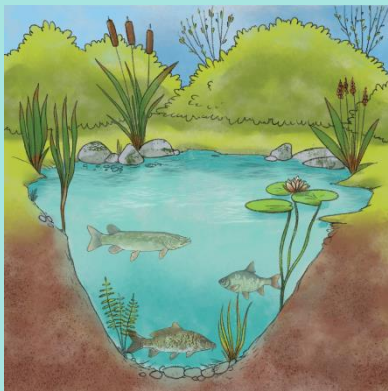
Phytoplankton samples are collected along a depth gradient, using an integrating water sampler. The sample is preserved and stained with a Lugol-fixative for storage until analysis. Under a light microscope, the cells of phytoplankton species are counted and the cell volume of each species is recorded ranging from $2 \mu\text{m}^3$ to $50\,000 \mu\text{m}^3$.

Based on cell biovolume and abundance, the total phytoplankton biovolume in the sample can be determined. Using the species composition, abundance, total cell biovolume, chlorophyll a concentration and indicator values of individual species, we calculate the multi-metric index of phytoplankton and the ecological status, which is mostly related of the nutrient loads to the lakes (trophic state).



Fish

Fish are often the final link in the food chain in waters, so they are an indicator of the state of the entire aquatic ecosystem. Due to their longer lifespan, they are sensitive to long-term changes in the environment. Fish reflect the state of the watercourse upstream as well as downstream and the state of tributaries, as they actively move during different life periods or parts of the day due to migratory patterns and the use of different habitats. Therefore, they are particularly sensitive to hydromorphological changes. Sampling is done by electric fishing in rivers and lakes and by using gill nets with different mesh sizes in the pelagial and littoral zones of lakes. Electric fishing is carried out by trained fishermen, which use electrical pulses, to temporarily stun the fish and get them out of the water. The fish are then counted, the species identified and their length and weight measured. After the measurements, the stunned fish are moved to a tub of fresh water, where they can recover before they get released into a quiet area near the banks of a river or lake. Gill nets are used to catch fish species that are unlikely to be caught with electrofishing due to their pelagic or benthic habitat preferences. Unfortunately, this is a very invasive approach and most fish caught with nets, die.



Fish reflect the state of the watercourse upstream as well as downstream and the state of tributaries. They are particularly sensitive to hydromorphological changes in water.

Based on the sampled area, number and data on the length and weight of fish, we calculate the abundance of the species and biomass per hectare of watercourse. By measuring the length, we get an insight into the age composition thus the reproductive success of the fish community. Based on the collected data, we calculate the appropriate indices and ecological status for general water degradation.



Ecological status & environmental DNA analysis

All organisms in the water leave their genetic traces, which contain key information about the ecological state of the environment. Researchers are working to learn to read these traces by sequencing environmental DNA (free DNA and DNA in microorganisms).



DNA (deoxyribonucleic acid) is a carrier of genetic information in all living things.
eDNA (environmental DNA) is all the genetic material found in an environment.

All analyzes begin with sampling. For the purposes of molecular analyzes, we need a sample of water or biofilm (a set of microorganisms attached to a solid substrate, e.g. phytobenthos) from which we isolate environmental DNA. Many organisms are present in the aquatic environment, from viruses and bacteria to plants and aquatic animals, as well as occasional visitors - humans or their pets. All of these organisms leave their DNA molecules in the environment. By the term "environmental DNA" (eDNA) we mean the entire hereditary material of all organisms that are (or have been) present in the sampled environment. This genetic material can be derived directly from the cells of microorganisms that are sampled along with water (e.g., microscopic algae or bacteria). In larger organisms (e.g. fish or humans), it is transmitted to the environment through body secretions, dead skin, hair and the like, and can last, in the form of free DNA molecules, in the aquatic environment for several days or even weeks. However, if DNA is trapped in sediments at the bottom of water bodies, it can remain there for years or decades; in some cases even millennia, which opens the door to paleoecological research.

The environmental DNA obtained from the water sample provides a number of answers to ecological questions; we just need to know how to read them. DNA is a long chain of nucleotides; symbolised by the individual letters A, T, G and C, which represent four different nitrogen bases (adenine, thymine, guanine and cytosine). The genetic alphabet therefore consists of only four letters that codify all life on our planet. If a single nucleotide is a single letter, the individual codons (sets of three nucleotides) are words, and each gene is its own sentence. Every organism, carries within it, a book called the genome - its entire genetic record.



This book parable, derived from the book *Genome* by author Matt Ridley, will help us understand the genetic record and molecular methods.

If we read the entire genome of each organism, we could recognize its identity; species, subspecies, sometimes also geographical origin or other characteristics. However, reading the entire genome (known as genomics) is time consuming and, above all, leads to huge amounts of data. Many different species of organisms can be expected in each sample. If we wanted to read the entire genomes of all the organisms present in each sample, processing and storing such a large amount of data would be a huge challenge.



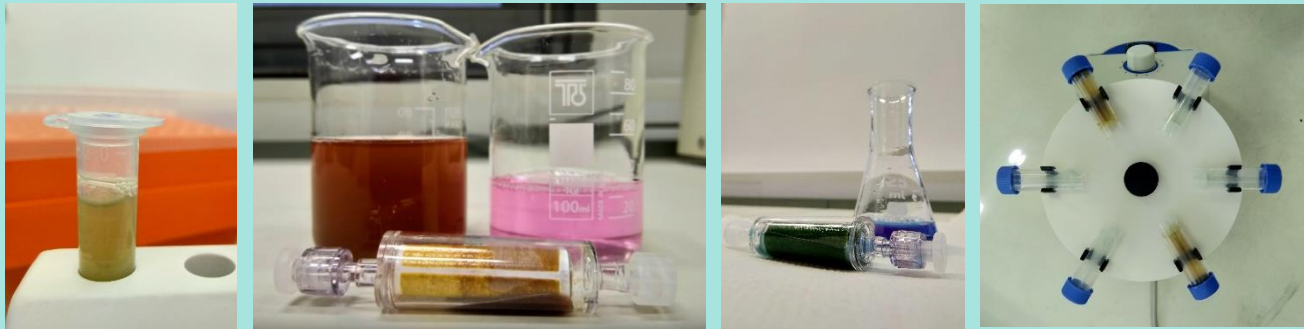
We are not interested in determining the ecological status of all organisms in water, but only selected groups of organisms. Instead of reading a book from beginning to end, we read only a short section of it - one that is unique to each type.

In addition, we are not interested in determining the ecological status of all organisms in water, but only selected groups of organisms. Instead of reading a book from beginning to end, we read only a short section of it - one that is unique to each type. For each group of organisms, a specific region of the genetic record was selected, on the basis of which we can distinguish individual species from each other. For bacteria, for example, this is one part of the 16S rRNA gene, that carries the transcript for the minor ribosome subunit. The sequence of nucleotides in this region must be sufficiently diverse between different species to allow distinction between them, while at the same time being preserved within the same species so that all its representatives can be identified as the same species. The term barcode has become established for such regions, which is why the process of reading the nucleotide sequence of selected DNA segments is also called barcoding.



Molecular laboratory library and printing house

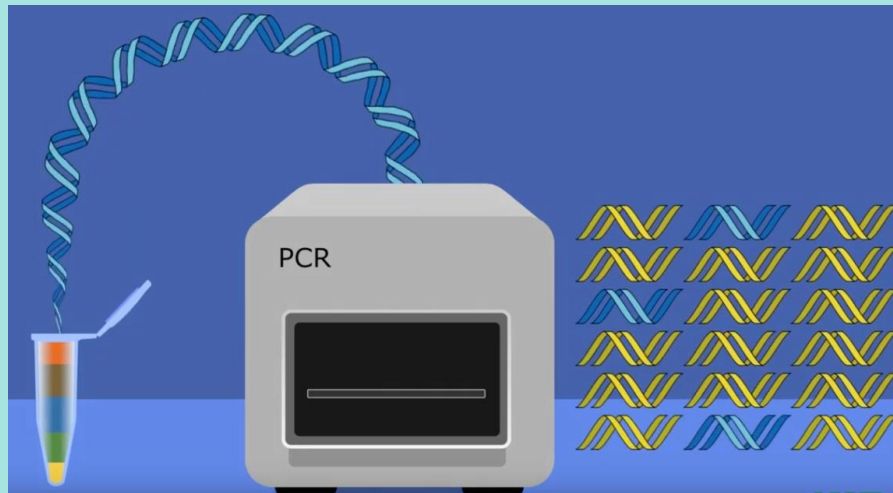
It is not a classic library where we borrow books, but a laboratory library where we look for specific phrases and then "multiply" or "print" them. Once we have selected the appropriate region for the organisms of interest, we need to multiply this section of DNA into as many copies as possible. In a sample containing the complete genetic records of all organisms present, namely, our selected section represents a negligibly small proportion in the multitude of millions and millions of nucleotides, so it cannot be analyzed separately. Imagine that you would have to find a specific phrase from a pile of thick books in each of them, without the "search" command, identify the differences in those phrases between individual books, and compile a report from your observations. Sounds like a long and arduous task.



Parts of a molecular laboratory library and printing house.

Researchers use a molecular tool that combines the "search", "copy" and "paste" commands - this is called polymerase chain reaction (PCR) and can create billions of copies in an hour or two from a few copies of our studied region of DNA. It does this by mimicking the same conditions that occur during DNA amplification in a living cell. The key role in this is played by short fragments of the nucleotide sequence, which perform the role of the "search" function. Because they are designed to match the first and last few letters of the phrase we are looking for, they will read only the selected part throughout the book and ignore any other unnecessary information. The other major player in multiplication is the enzyme polymerase, which performs the functions of "copy" and "paste", just as happens right now in the cells of your body. The only difference is that in living cells, when they divide, the entire hereditary record is doubled, whereas in a laboratory reaction, only a selected region.

After such an amplification reaction, our studied region of DNA represents the vast majority in the sample, and the remaining DNA only a negligible proportion.



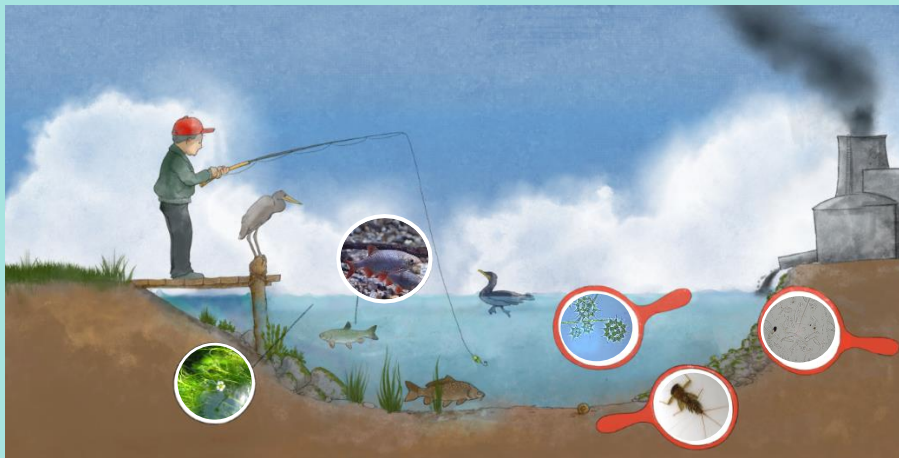
Multiplication of selected (yellow) DNA regions.

Now, instead of a thick book full of information useless to us, we have a folder full of identical copies of the phrase we are interested in. We just need to read this phrase - our chosen section of DNA - in a process called DNA sequencing. After DNA sequencing, we have before us a huge number of such letters that make up the sequences of individual species. However, since - as in a foreign language - the sequence of letters alone means nothing to us, we need a dictionary. Here they enter the story of the reference library; extensive biological databases where the nucleotide sequences of many species of organisms obtained by DNA sequencing as part of research projects are stored. The translation of a nucleotide sequence into lists of species of organisms takes place using various algorithms; as if you were typing a foreign word into a browser in an online dictionary, which would return its meaning to you in a form you understand. When this is done for all nucleotide sequences read, the result is a list of all identified species of organisms present in the sample. However, there are still many species of organisms that do not yet have their own entry in reference libraries; we do not know their genetic record. This occurs mainly in those microscopic organisms whose cultivation and identification in the laboratory is very difficult or impossible. Such shortcomings in reference libraries greatly complicate the bioinformatic processing of barcoding results.



The future of assessing the water status

The greatest potential of so-called molecular monitoring lies in saving time, increasing the sensitivity of methods, detecting species without harming them (e.g. fish) and the possibility of reducing costs in the future. We can imagine what an effort it is to carry out a full monitoring of the fish by placing nets at all depths in the lakes compared to filtering a few liters of water. DNA sequencing technology is evolving rapidly, and the cost of the process is falling. Compared to time-consuming determination of indicator species based on morphological criteria, molecular methods are highly powerful and allow parallel analysis of many samples in a short time. In addition, they could also make it possible to detect rare species that are difficult or even impossible to detect with traditional methods.



Indicator species are found within all biological elements for determining the ecological status of waters by the traditional method: phytoplankton, phytobenthos and macrophytes, benthic invertebrates and fish. Molecular tools suggest that indicator species can also be found in other groups of organisms. The aquatic ecosystem is much more complex than can currently be detected by traditional methods.

Of course, the inclusion of new DNA-based methods in monitoring practices is not straightforward. Monitoring of ecological status is based on long time series of data, for the comparison of which it is crucial that they were obtained using the same methodology. In addition, a reference condition is defined for each type of water body - one where no human impact is observed or is very small - which serves as a baseline for determining quality. The transition to molecular monitoring therefore requires time to ensure a successful implementation in routine biomonitoring. It is first necessary to systematically evaluate the



reliability of these methods and the comparability of their results with the results of traditional methods, to adapt existing biological indices and reference conditions and to adjust sampling procedures to meet the needs of molecular analyzes after aseptic field and laboratory treatment of biofilm and water samples.

Many research projects and networks of scientific institutions around the world are focusing on these issues, and some countries have already begun to introduce DNA barcoding into their water ecological monitoring program. The first European country to take this step was the United Kingdom; a few years ago it announced the start of molecular monitoring for a group of diatoms that are one of the key bioindicators in aquatic environments. However, the introduction of such changes requires caution and forethought, as the ambiguities already mentioned are key to the gradual introduction of new methods, which should primarily complement and not completely replace the approach established so far. Molecular monitoring has not yet been established in all Alpine countries, but research is already underway to show that it is very useful for traditional and molecular monitoring to be carried out in parallel for some time.

Molecular methods have great potential in monitoring the ecological status of water, and only time and numerous studies will show how we can use them most meaningfully. But life (water) will be more understandable after updating our conventional monitoring methods. The short-term goal of the researchers is to establish the most optimal strategy for all types of water bodies and for all biological elements, which will achieve a comprehensive insight into the actual state of the ecosystem with the lowest investment of time and money through a combination of traditional and molecular methods. It should be noted that molecular methods are unlikely to ever completely replace traditional ones and that the need for qualified species morphological experts will remain present for a long time to come.

We must not forget that these are new tools, but still the main goal is:
to protect our water resources and provide clean water
to the entire population in the future as well.



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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