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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Deliverable D.T3.2.3

Evaluation of the Ecological Status of waterbodies by using traditional approaches complemented by innovative tools.

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1 Lake Mondsee, Austria

1.1 Phytoplankton (incl. cyanobacteria)

Rainer Kurmayer, Hans Rund, Josef Wanzenböck

Ecological status according to national legislative

The relevant metric EQR (Ecological Quality Ratio) is derived from Chlorophyll a concentration, phytoplankton total biovolume and the Brettum index calculated from trophic indeces of indicative phytoplankton taxa. Phytoplankton taxa are standardized using the REBECCA code (Ofenböck et al. 2016).

For Mondsee the EQR based on Chlorophyll varied between 0.47 – 0.85, for Phytoplankton biovolume between 0.56-0.81, for the Brettum Index from 0.64-0.8 (Table 1). Overall the ecological quality was estimated as **good** when compared to the natural reference situation. Typically the EQR ranges between the thresholds of ”good” and “very good”. Only in October 2019 the ecological quality was found decreased and estimated as “moderate”, which mas mostly because of a significant increase in Chlorophyll a concentration, i.e. a maximum Chl. a concentration of 6 µg/L was recorded. The Brettum-Index mainly is derived from diatom taxa like *Aulacoseira subarctica/islandica*, *Cyclotella cyclopuncta*, *C. radiosa*, *Stephanodiscus neoastrea*, *S. minutulus*, and *Ulnaria ulna (var. delicatissima*) which are indicative of oligomesotrophic conditions. In addition, the metalimnetic growing cyanobacterium *Planktothrix rubescens* is indicative of mesotrophic conditions.

Table 1: Normalized Ecological quality ratio (nEQR) derived from Phytoplankton sampling using the standard technique (Ofenböck et al. 2016) for Lake Mondsee (from Chlorophyll a concentration, phytoplankton total biovolume and the Brettum index calculated from trophic indices of indicative phytoplankton taxa) during January 2019 – 2020 (n=13).

	Chlorophyll-a	Biovolume	Brettum-Index	nEQR total	Status
Jän.19	0.83	0.80	0.68	0.75	good
Feb.19	0.79	0.75	0.69	0.73	good
Mär.19	0.62	0.60	0.70	0.65	good
Apr.19	0.72	0.56	0.69	0.66	good
Mai.19	0.77	0.67	0.80	0.76	good
Jun.19	0.85	0.74	0.77	0.78	good
Jul.19	0.69	0.68	0.69	0.69	good
Aug.19	0.69	0.70	0.67	0.68	good
Sep.19	0.61	0.78	0.67	0.68	good
Okt.19	0.47	0.60	0.64	0.59	moderate
Nov.19	0.61	0.60	0.68	0.64	good

Dez.19	0.56	0.60	0.68	0.63	good
Jän.20	0.83	0.81	0.68	0.75	good

Additional information through HTS (high throughput sequencing)

1) By HTS the occurrence of relevant taxa to calculate the Brettum index was confirmed, most importantly from Cyanobacteria (*Planktothrix, Aphanizomenon, Snowella*), diatoms (*Aulacoseira subarctica, Ulnaria ulna*), haptophytes (*Chrysochromulina parva*), green coccale algae (*Botryococcu*s) and dinophytes (*Peridinium willei*), see Suppl Table 1.1 (in D.T3.2.1). For specific (abundant) cyanobacteria such as *Planktothrix* a good quantitative relationship between HTS read numbers and *Planktothrix* biovolume was observed.

2) For L. Mondsee new relevant phytoplankton taxonomic information includes mostly picocyanobacteria, i.e,. the genera *Synechococcus* and *Cyanobium* which are hardly quantitatively detected via microscopical methods. Aside from *Planktothrix* any other nuisance cyanobacteria (i.e. *Microcystis aeruginosa, Tychonema bourellyi*, *Dolichospermum lemmermanni*) were recorded in L. Mondsee.

3) The unequivocal identification of phytoplankton taxa is critical for monitoring the toxigenic basis of algal growth. For example, in contrast to *Planktothrix rubescens/agardhii* carrying the microcystin biosynthesis genes very frequently, the genera *Snowella* and the picocyanabcteria composed of *Cyanobium* sp. or *Synechococcus* sp. have not been shown to carry the respective cyanotoxin synthesis genes.

4) Under decreasing ecological status (from good to moderate) cyanobacteria of the genus *Planktothrix* declined at the expense of (faster growing) unicellular cyanobacteria, i.e. mostly *Snowella* and *Cyanobium* sp. The ecological conditions favouring this change rather might imply a change in mixing and irradiance conditions in the water column rather than an environmental deterioration.

1.2 Littoral Phytobenthos

not in use (not applicable)

1.3 Fish composition

Hans Rund, Josef Wanzenböck, Rainer Kurmayer

Ecological status according to national legislative

During the last WFD assessment in 2010, using traditional methods (gillnetting and electrofishing), 19 different fish species were detected and the ecological status of fish was rated **good** (Gassner et al. 2013).

Additional information through HTS (high throughput sequencing)

+) All species that were detected during the sampling event in 2010 were also detected with the eDNA approach carried out in 2019. Thus the 19 different fish species occurring in L. Mondsee were confirmed.

+) In addition, signals indicative of 6 species such as *Cottus gobio* (0.9%), *Cyprinus carpio* (0.4%), *Onchorynchus mykiss* (0.4%), *Scardinius erythrophtalmus* (0.3%), *Barbus barbus* (0.1%) and *Thymallus thymallus* (0.01%) were found in very low proportions.

+) Cottus gobio (0.9%), *Barbus barbus* (0.1%) and *Thymallus thymallus* (0.01%) occur in some of the tributaries of Lake Mondsee and historical records report catches of these species in the lake from time to time.

+) Onchorynchus mykiss (0.4%), *Cyprinus carpio* (0.4%) and *Scardinius erythropthalmus* (0.3%) are considered non-native, however several catches of these species have been reported. These findings were confirmed by the molecular approach.

+) Taking the eDNA results into consideration, the ecological status of fish in Lake Mondsee would decrease due to the introduction of additional non-native fish. Nevertheless, the ability to simultaneously detect species from the tributaries when actually only the lake is sampled emphasizes the potential of eDNA sequencing for biodiversity assessment in the catchment. Thus this methodology would be relevant for biodiversity assessments at a larger scale, e.g. the entire Natura 2000 Mondsee - Attersee region.

2 Lake Bourget, France

France (PP6, INRAE; PP11, OFB)

2.1 Phytoplankton (incl. cyanobacteria)

Isabelle Domaizon, Marine Vautier, Frederic Rimet

Ecological status according to national legislative

The trophic level of lakes can be assessed using indices based on taxonomic composition and phytoplankton biomass.

Studies showed that the **Brettum index** (modified by Wolfram et al. 2007, Wolfram & Dokulil 2007) was the most suitable for assessing the trophic level of large alpine lakes (Kaiblinger 2008, Anneville & Kaiblinger 2009, Kaiblinger et al. 2009), and this index is then used to monitor the lake Bourget.

Another index is calculated in France, the **IPLAC index** (Performance of the Phytoplankton Index for Lakes). It is a multimetric phytoplankton index to assess the ecological status of water bodies in France. The index is WFD-compliant and is dedicated to natural lakes and artificial water bodies in metropolitan France, and is routinely used by the French Ministry of the Environment to assess lake ecological status through the phytoplankton community. The IPLAC is a multimetric index, taking into account biomass, abundance and species composition of communities. The first metric is based on the total phytoplankton biomass (MBA), the second on the abundance and taxonomic composition (MCS) (Laplace-Treyture., 2016).

Table 1: *Chlorophyll a concentration, phytoplankton total biovolume, Brettum and IPLAC indices calculated from trophic indices of indicative phytoplankton taxa during January 2019 – December 2019 (n=12). Data published in the scientific report Jacquet et al., 2020.*

Chlorophyll-a	Biovolume	Brettum-index	IPLAC-index
3 µg.L^{-1}	1 510 µg.L^{-1}	4.416	0.735

The functional indexes of Brettum (4.416) and IPLAC (0.735) show a **good global state** of the pelagic ecosystem of the Lac du Bourget for the year 2019.

The Brettum-Index is mainly derived from taxa which are indicative of oligomesotrophic conditions. In addition, some species indicative of oligotrophic environments are also present in 2019 with important biomasses (e.g. *Epipyxis polymorpha*, *Chrysolykos planktonicus*, *Kephyrion* and *Pseudokephyrion*).

Additional information through HTS (high throughput sequencing)

1) Some species that are difficult to differentiate by morphology under microscopy (e.g. small *Chlorella* like species which can belong to completely other algal classes such as *Eustigmatophyceae*) are unambiguously distinguishable by HTS. The identification of these species in morphology is subject to biases due to the observer who will assign these species according to his/her knowledge of the environment and/or other surrounding species, whereas with HTS the identification will be unbiased. There is, however, a limitation in HTS which is the completeness of the molecular databases.

2) For Lake Bourget, new relevant phytoplankton taxonomic information are revealed, including picocyanobacteria, as the genera *Cyanobium*, which are hardly detected via microscopical methods.

3) The correspondence between genus identified by microscopy and HTS is good (see Deliverable D.T3.2.1.), but species assignment and relative abundances vary substantially between the two methods. Thus, there is still development to be done for phytoplankton indexes from HTS data, but the potential is high.

2.2 Littoral Phytobenthos (benthic diatoms)

Not applied

2.3 Fish composition

Marine Vautier, Chloé Goulon, Jean Guillard, Maxime Logez, Jean-Marc Beaudoin, Isabelle Domaizon

Ecological status according to national legislative

A WFD assessment using traditional methods (gillnetting) was conducted two weeks before the eDNA monitoring in October 2019. The traditional monitoring allowed the identification of 19 different fish species in lake Bourget. The ecological status of the lake Bourget fish population in 2019 has been defined as **very good** (scientific report Jacquet et al., 2020).

Additional information through HTS (high throughput sequencing)

+) 18 of the 19 species that were detected during the traditional sampling event were also detected with the eDNA approach carried out in 2019.

+) *Carassius carrassius*, was detected during the traditional monitoring (3 fishes), but not with the eDNA approach, suggesting that the eDNA method used is not optimal for all the rare species

+) In addition to species identified with traditional monitoring, 9 species were identified only with the eDNA method, as *Leuciscus leuciscus*, *Barbatula barbatula*, *Salmo trutta or Salvelinus alpinus* (see D.T3.2.1 for details). All species identified with eDNA only have already been identified with traditional approaches over the previous 25 years. There is therefore no identification of new species, but confirmation that species present in a low level are still present in the lake, such as *Barbus barbus* or *Cottus gobio*.

+) Apart from one rare species that was not identified with eDNA, the eDNA approach identified more species that had not been identified by traditional methods for several years, and the ecological status of the lake Bourget would therefore likely to be increased with an eDNA approach. The ecological status of the Lake Bourget fish population in 2019 is very good and would remain very good with ADNe monitoring. Thus this methodology would be relevant for biodiversity assessments of French alpine lakes.

3 Lake Starnberg, Germany

3.1 Phytoplankton (incl. cyanobacteria)

Ute Mischke

Ecological status according to national legislative

In Germany the Phyto-See-Index (PSI) has been used since 2008. The European Commission has published the agreed classification systems of the member states – including the Phyto-See-Index - as the result of the intercalibration process in the Official Journal of the European Union (EC 2008, 2013). The assessment of a lake is strongly dependent on its lake type. Classification by lake type takes into account a lake's theoretical trophic status under minimal anthropogenic influence, its "reference state". For Lake Starnberg the reference state is oligotrophic and according lake type PP1 "Natural, artificial and heavily modified Alpine lakes, calcareous, dimictic".

The Phyto-See-Index (PSI) is a multimetric index and is calculated with a tool (PhytoSee; Mischke et al. 2017), taking into account biomass, algal class metrics (share of dinoflagellates n %; diatom-, crypto and-cyanopyhtes biovolumes) and species composition of communities (PTSI, Mischke et al. 2016).

The PSI assesses a **good ecological state** of phytoplankton in Lake Starnberg for the year 2019.

Table 1: *PSI and single metrics calculated based on phytoplankton analysis during March 2019 – October 2019 (n=8) and mean values of chlorophyll a concentration, phytoplankton total biovolume in lake Starnberg.*

PSI	Total ES class	biomass metric	algal class metric	PTSI metric	biovolume	Chlorophyll a
1,58	**good**	1,77	1,32	1,51	0,6922 µg.L-1	2,4µg/l

In total 59 different phytoplankton taxa were detected by light microscopy, from which 7 centric diatoms were identified on species level only by the help of the routinely prepared diatom slides (see table 2).

Table 2: List of indicator taxa of the phytoplankton metric PTSI which were found by light microscopy (LM) but not by HTS (16S, 18S, rbcL) in lake Starnberg.

LM_order	LM_Taxon_REBECCA	LM_ID-REBECCA
Pennales	Fragilaria crotonensis	R0223
Pennales	Cocconeis placentula	R0155
Centrales	Cyclostephanos delicatus	R2109
Centrales	Cyclostephanos dubius	R0038
Centrales	Cyclotella balatonis	R1EAW
Centrales	Cyclotella comensis	R0042
Centrales	Cyclotella ocellata	R0048
Centrales	Discostella stelligeroides	R2511
Centrales	Discostella glomerata	R2058
Centrales	Stephanocostis chantaica	R0075
Centrales	Stephanodiscus minutulus	R0082
Centrales	Stephanodiscus alpinus	R0076
Centrales	Stephanodiscus neoastraea	R0083

Additional information through HTS (high throughput sequencing)

1) Species, which are difficult to differentiate by morphology under microscopy (e.g. small Chlorella - like species, and other spherical small cells partly get loose from colonies by fixation), can belong to various algal classes. With HTS the identification is strongly improved: In lake Starnberg we found HTS signals for *Cyanobium, Radiocystis, Snowella, Woronichinia, Synechococcus,* and *Choricystis*.

2) For Lake Starnberg, new relevant phytoplankton taxonomic information are revealed by HTS, especially for dinoflagellates and chrysophytes. For example, species as *Asulcocephalium miricentonis, Chrysosphaerella sp., Paraphysomonas vestita* and *Hafniomonas reticulate*, are hardly to detect via microscopical methods.

3) In case of Bavarian plankton samples, the project partner requested a special additional work: The gen marker rbcl for diatoms was also applied and 82 taxa (mainly Pennales) were detected more than found by microscopy in the 46 plankton samples from Bavarian lakes. On the other hand, important centric diatom indicators were missing in the signal list for lake Starnberg (see table 2). The molecular databases obviously not cover all indicators.

4) The correspondence between genus identified by microscopy and HTS is good (see Deliverable D.T3.2.1.), but species assignment and relative abundances vary substantially between the two methods. Thus, there is still development to be done for phytoplankton indices based on HTS data.

3.2 Littoral Phytobenthos (benthic diatoms)

Ecological status according to national legislative

The WFD requires monitoring of macrophytes and phytobenthos (including diatoms) for the assessment of the ecological quality of lakes, which is done by the German PHYLIB assessment method.

Here only the assessment based on benthic diatoms is reported.

The classification of lakes from diatoms is based on the German Diatom index based on the recorded species by light microscopy and the attribution of trophic weights of the found species (TI) and the proportion of reference taxa for the given water body type.

The Diatom index and revealed in total a good ecological status class on average of the 27 transects from the littoral shore stations of the oligotrophic Lake Starnberg.

Additional information through HTS (high throughput sequencing) for the German diatom index within the PHYLB assessment

Diatom Index with the addition of the new contribution of the HTS species was not applied because these new species haven't the relative trophic weights in the reference list. The Index requires that 95% of species are determined on species level, and the calculation tool handle the "unknown", new detected HTS taxa like insufficient determination.

The additional information on the occurrence of invasive taxa such as *Achnanthidium delmontii* and *Achnanthidium eutrophilum* become available, which are new for Bavarian region. In total, 51 diatom taxa were detected by HTS but not by light microscopy.

An examination of in total 45 diatom taxa on the scanning electronic microscope (SEM) was ordered in the Eco-AlpsWater with the intention to find out whether confusion of similar species had taken place during the light microscopic evaluation (Goos 2021), in samples when taxa detection was missing with one method. For this SEM study, exactly the same diatom suspensions from project samples were uses, which have been analysed by HTS and by light microscopy.

For example, during the genetic survey, a strong signal of *Encyonopsis minuta* was found in the Lake Starnberg transect 21. Also, *Encyonopsis microcephala* and *Encyonopsis subminuta* were found as well as Encyonopsis spec.

In the SEM *Encyonopsis microcephala* was dominant. *Encyonopsis minuta* strongly resembles this species and was probably therefore overlooked in the light microscope. With the help of the SEM, a clear differentiation was much easier, and its detection was easier with HTS. A confusion of the above-mentioned species has no effect on the ecological classification of the water bodies, as they all have the same trophic and saprobic values.

3.3 Fish composition

Michael Schubert, Christian Vogelmann

Ecological status according to national legislative

Through the traditional sampling at Lake Starnberg in autumn 2019, 26 species were detected and the ecological status, based on the DeLFI Site Modul (Ritterbusch et al. 2015) was assessed as good.

Additional information through HTS (high throughput sequencing)

VigiDNA®:

+) In total 24 species could be detected with the VigiDNA® System. Of the total 26 species detected in Lake Starnberg by traditional methods, 20 species could be confirmed using the eDNA approach (VigiDNA®).

+) In addition, signals indicative for *Barbus barbus* (0.16%), *Carassius gibelio* (0.11%), *Gymnocephalus cernua* (0.16%), *Salvelinus fontinalis* (0.12%), *Sander lucioperca* (0.06%) were found in very low proportions.

+) Alburnuides bipunctatus (1.48%), Gobio gobio (0.48%) and Gymnocephalus cernua (0.16%) occur in catchment area of Lake Starnberg*. The detections of Ctenopharyngodon idella (0.44%). could originate from fishing gear or from nearby ponds draining into Lake Starnberg.*

GFC:

+). Using the eDNA approach (GFC filter), a total of 23 species could be detected. 20 of the 24 traditionally confirmed species could be detected, in addition *Onchorynchus mykiss*, *Salvelinus fontinalis*, *Salmo trutta* and *Thymallus thymallus* were detected in small proportions.

+) *Leuciscus idus* and *Leuciscus leuciscus* could not be separated from each other.

+) Signals from *Barbartula barbarula* (0.01%) and *Brown trout* (0.01%) were found in very low proportions.

+) The original native *Phoxinus phoxinus* could not be detected by both the traditional and eDNA approaches.

+) Taking the eDNA results into consideration, the fish based ecological assessment of Lake Starnberg would not change. Nevertheless, the ability to simultaneously detect species from the tributaries when actually only the lake is sampled emphasizes the potential of eDNA sequencing for biodiversity assessment in the catchment. Thus, this methodology would be relevant for biodiversity assessments at a larger scale.

4 Lake Garda, Italy,

Italy (PP3, ARPAV)

4.1 Phytoplankton (incl. cyanobacteria)

Giorgio Franzini, Chiara Zampieri, Federica Giacomazzi, Giampaolo Fusato

Ecological status according to national legislative

The relevant metric EQR (Ecological Quality Ratio) is derived from annual mean Chlorophyll a concentration, annual mean phytoplankton biovolume and the annual PTIot index calculated from indicative phytoplankton taxa trophic indices. Phytoplankton taxa are standardized using the REBECCA code (Ofenböck et al. 2016).

For Lake Garda the EQR was based on annual mean Chlorophyll concentration of 2.13 mg/m3, annual mean phytoplankton biovolume of 2.32 mm3/m3 and annual PTIot of 2.84.

The ecological quality was estimated as **moderate** when compared to the natural reference situation.

Typically the EQR ranges between ”moderate ” and “good” during the years.

The moderate ecological status was mainly determined by the biovolumes of *Mougeotia sp*.(that in 2019 was particularly high), the cyanobacteria *Planktothrix rubescens* and *Tychonema bourrellyi*, and the diatoms *Fragilaria crotonensis*, *Aulacoseira granulata var. angustissima*, *Cyclotella spp* and *Stephanodiscus sp*.

Additional information through HTS (high throughput sequencing) for the PTIot index

1. HTS taxa without a corresponding PTIot score were not considered
2. Thanks to HTS the haptophyta *Chrysochromulina*, both *C. parva* and sp., and the chlorophyta *Chlamydomonas reinhardtii* were detected
3. The cyanobacteria *Radiocystis geminate* and *Synecococcus* were also detected. We are processing data about the picocyanobacteria in HTS and LM databases.
4. Biovolume values are very relevant for the PTIot index, and HTS abundance (reads) are not directly usable to calculate index. We didn't find a good quantitative relationship between HTS reads and observed taxa biovolume. In our samples the possible reasons are that the cellular/colonial taxa biovolumes depend on seasonality, in addition to the number of cells/colonies.
5. We tried to convert HTS reads in biovolume, with elaboration between LM and HTS data, to apply PTIot index with the additional HTS taxa. In every data processing attempt the PTIot value indicated a worse ecological status of the lake (but always in moderate class).

4.2 Littoral (Benthic Diatoms)

Ecological status according to national legislative

The WFD requires monitoring of macrophytes and phytobenthos (including diatoms) for the assessment of the ecological quality of lakes.

The classification of lakes from diatoms is based on EPI-L index based on the recorded species and the attribution of trophic weights of the found species.

Additional information through HTS (high throughput sequencing) for the EPI-L index

EPI-L Index with the addition of the new contribution of the HTS species doesn't work because these new species haven't the relative trophic weights in the reference list. The Italian EPI-L Index requires the 70% of species with trophic weights inside this reference list.

4.3 Fish composition

Not applied

5 Lake Bled, Slovenia,

Slovenia (PP5, ARSO)

5.1 Phytoplankton (incl. cyanobacteria)

Špela Remec-Rekar, Katarina Novak

Ecological status according to national legislative

During the intercalibration of biological methods in the frame of the WFD implementation, Slovenia adapted AT methodology for the ecological status assessment with phytoplankton (Wolfram G. et al., 2009). Multimetric phytoplankton index (MMI_FPL) derived from chlorophyll a concentration, phytoplankton total biovolume, and the Brettum index calculated from trophic indices of indicative phytoplankton taxa is the relevant EQR value for the ecological status assessment in lakes. Phytoplankton taxa are standardized using the REBECCA code.

For the Lake Bled, the EQR based on chlorophyll varied between 0.41 – 1.00, phytoplankton biovolume between 0.19-0.65, and Brettum Index from 0.51-0.8 (Table 1). The ecological quality was estimated as slightly moderate when compared to the natural reference situation.

The Lake Bled phytoplankton EQR values range mostly between ”moderate” and “good”. However, especially during the mixing period, when autochthonic nutrient sources become available, total phytoplankton biovolume and chlorophyll a could also be ranged into poor ecological class. On the other hand, in June 2019, the ecological quality increased. Both quantity phytoplankton parameters showed their year minimum and were estimated as “good” and “very good” (Biovolume; minimum 0,89 mm3/L and chlorophyll a concentration; minimum 1,4 µg/L). Brettum index also expresses “very good” status due to the dominance of *Cyclotella cyclopuncta* = *Cyclotella costei*, an indicator of oligotrophic conditions and almost completely diminished *Planktothrix rubescens* population in June. *Planktothrix rubescens* indicates mesotrophic conditions, and its dominance greatly influenced a “moderate” status of Brettum index during both mixing periods.

Since 1980/81, Lake Bled has been under the intensive reoligotrophication process initiated by increased hydrological inflow (artificial inflow of River Radovna) and hypolimnetic outflow (siphon- Olszewski tube). Both applied restoration measures with management supported by the lake monitoring enable the epilimnetic and hypolimnetic rinsing of the lake with considerable influence on its lower trophic status.

Table 1: Normalized Ecological quality ratio (nEQR) derived from the chlorophyll a concentration, phytoplankton total biovolume, and the Brettum index calculated from trophic indices of indicative phytoplankton taxa in the Lake Bled from January to December 2019 (n=12). Phytoplankton sampling using the standard technique from the official SI phytoplankton methodology “Metodologija vrednotenja ekološkega stanja jezer na podlagi fitoplanktona”. https://www.gov.si/assets/ministrstva/MOP/Dokumenti/Voda/Ekolosko_stanje/

2019	Biovolume	Chlorophyll a	Brettum-Index	MMI_FPL	Status
	nEQR	nEQR	nEQR	nEQR total	
Jan.	0,41	0,43	0,68	0,55	moderate
Feb.	0,20	0,43	0,68	0,51	moderate
Mar.	0,46	0,41	0,67	0,55	moderate
Apr.	0,19	0,43	0,68	0,50	moderate
May	0,40	0,65	0,73	0,63	good
Jun.	0,65	1,00	0,80	0,81	very good
Jul.	0,61	0,42	0,69	0,60	good
Aug.	0,55	0,79	0,54	0,61	good
Sep.	0,62	0,91	0,51	0,64	good
Oct.	0,51	0,82	0,60	0,63	good
Nov.	0,49	0,43	0,67	0,57	moderate
Dec.	0,33	0,22	0,65	0,47	moderate
Average	**0,45**	**0,58**	**0,66**	**0,59**	**moderate**

Additional information through HTS (high throughput sequencing)

1) By HTS, the occurrence of relevant taxa to calculate the Brettum index was confirmed, most importantly from Cyanobacteria (*Planktothrix, Aphanizomenon, Anabaena - Dolychospaermum*), haptophytes (*Chrysochromulina parva*), green coccale algae (*Botryococcus*), and dinophytes (*Peridinium willei*) see Suppl Table 5.1 (in D.T3.2.1). For specific (abundant) cyanobacteria such as Planktothrix a good quantitative relationship between HTS read numbers, and *Planktothrix* biovolume was observed.
2) For L. Bled, new relevant phytoplankton taxonomic information includes mostly picocyanobacteria, i.e., the genera *Synechococcus* and *Cyanobium,* which are hardly quantitatively detected via microscopical methods.
3) The unequivocal identification of phytoplankton taxa is critical for monitoring the toxigenic basis of algal growth. For example, unlike to Planktothrix rubescens/agardhii carrying the microcystin biosynthesis genes very frequently, the picocyanabcteria composed of Cyanobium sp. or Synechococcus sp. have not been shown to carry the respective cyanotoxin synthesis genes.

5.2 Phytobenthos (benthic diatoms)

Katarina Novak, Aleksandra Krivograd Klememčič

Ecological status according to national legislative

Phytobenthos is used as a quality element to assess the ecological status for several reasons: its sampling is simple, reacts to changes in water quality, and has a short generation time, which is why it responds to changes in the environment first. Phytobenthos is one of the biological quality elements based on which we evaluate the ecological status of watercourses. In Slovenia, only diatoms (Bacillariophyceae) are used for ecological status assessment. To assess the ecological status of watercourses based on phytobenthos, we assess the status only to one module - module of trophic - trophic index (TI) with which we see mainly the impact of eutrophication and changed land use in the catchment area of the watercourse.

Overall, the ecological quality ratio (EQR) for trophic state has been assessed as moderate at all ten sampling sites (T1-T10).

Additional information through HTS (high throughput sequencing)

+) For diatoms, correspondence between microscopy and sequencing is useful for confirming microscope-based identification of genera and species and thus rendering the expert assessment more reproducible.

5.3 Fish composition

Špela Remec-Rekar, Nataša Dolinar

Ecological status according to national legislative

During the last WFD assessment in 2018, using traditional methods (gillnetting and electrofishing), 9 different fish species were detected in **Lake Bled.** The ecological status of fish was rated **moderate** (G. Urbanič, S. Podgornik 2018).

Additional information through HTS (high throughput sequencing)

+) The majority of the species (7) detected during the sampling event in 2018 were also detected with the eDNA approach carried out in 2019.

+)Species ***Tinca tinca*** *(tench)* and ***Scardinius erythrophtalmus*** (common rudd) were not noticed by HTS

+)Species ***Thymallus thymalus*** (2%) and ***Salmo labrax*** (5.6%) were detected only with HTS. The origin of these signals is most likely the River Radovna - main lake inflow and fishkeeping farm on the lake tributary Mišca.

+) Species ***Cyprynus carpio*** (only one fish) was caught in 2018; only 0.02% of all fish caught. This seems unrealistic because carp fishing is the most popular sort of the sport fishing in Lake Bled. From the fishing and fishkeeping plans of the local Fish Club, it is clear that the annual input of the *Cyprinus carpio* into the Lake Bled is 200 kg, and from the report of Bled Fishing Club during the period 2000 – 2014, 6.972 adult carps were introduced.

+) family **Cyprinidae** noticed by HTS represent 23% of all fish e-DNA signals

+) Considering the eDNA results, the ecological status of fish in Lake Bled would decrease due to the realistic evaluation of alien species. Nevertheless, the ability to simultaneously detect species from the tributaries when only the lake is sampled emphasizes the potential of eDNA sequencing for biodiversity assessment in the catchment.

6 Lake Lugano, (CH-IT)

Switzerland (SUPSI)

6.1 Phytoplankton (incl. cyanobacteria)

Camilla Capelli, Fabio Lepori

Ecological status according to CIPAIS

The International Commission for the Protection of Waters between Italy and Switzerland (CIPAIS) set the objective to achieve to a “good” status for Lake Lugano (mesotrophy). Different parameters are measured, however CIPAIS identified, for some indicators, specific objectives to be pursued: Chlorophyll a concentration and phytoplankton composition (%cyanobacteria).

The optimal Chlorophyll a concentration (annual average) is set within a range of 2.5 to 4 µg/L. In 2019, the Chlorophyll a value in the productive layer was above this threshold (6.4 µg/L), which indicate mesotrophic conditions.

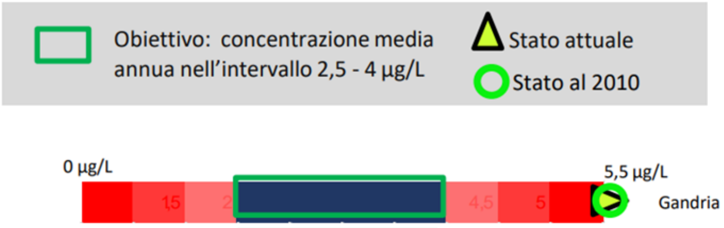

Fig. 1 CIPAIS Pannello di Controllo 2019, Lake Lugano, Chlorophyll a concentration compared to the objective.

A numerical quality objective for phytoplankton has not yet been defined for this indicator in Lake Lugano. However, the average annual biovolume of cyanobacteria is used as index of the ecological status. An average annual biovolume of cyanobacteria less than 28% of the average annual biovolume of the total phytoplankton community is used as an index of good ecological quality. In 2019, this value exceeded the threshold (35%), supporting that the current ecological status of Lake Lugano has not yet reached "good" conditions.

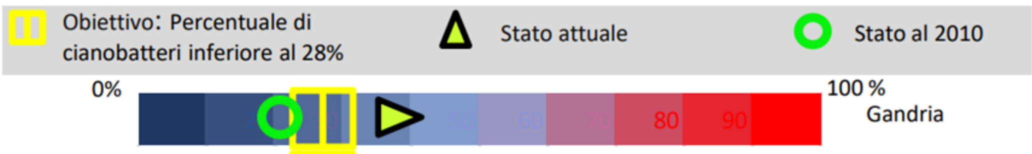

Fig. 2 CIPAIS Pannello di Controllo 2019, Lake Lugano, percentage of Cyanobacteria to total phytoplankton compared to the objective.

Ecological status according to ARPA

The environmental agency, ARPA Lombardia, define the ecological status of the Italian waters of Lake Lugano according to the metrics included in the Italian phytoplankton assessment method. The relevant metric EQR (Ecological Quality Ratio) is derived from the biomass metrics chlorophyll a, and total biovolume and the taxonomic composition of phytoplankton (PTIot - Phytoplankton Trophic Index). In 2019, the Italian waters of the North basin of Lake Lugano were included in the class "Moderate".

Additional information through HTS (high throughput sequencing)

For cyanobacteria, especially the abundant taxa such as *Planktothrix*, a good quantitative relationship between HTS read numbers and biovolume was observed. These results suggest that HTS can easily integrate traditional monitoring within the indicator regarding Cyanobacteria biovolume.

6.2 Littoral Phytobenthos (benthic diatoms)

Not appplied

6.3 Fish composition

Not appplied

7 River Steyr, Mondsee, Austria

Austria (PP2, LFUI)

7.1 Phytobenthos (incl. cyanobacteria)

Hans Rund, Josef Wanzenböck, Rainer Kurmayer

Ecological status according to national legislative

River Steyr is largely used for power generation purposes and as a recreation area. The relevant metric EQR is derived from the (i) trophic status module (assessing nutrient load), (ii) the saprobic status module (for organic load), (iii) the reference-species module (assesses the deviation of the found biotic community from the reference biocoenosis), Ofenböck et al. 2015. In general all modules have been rated with the quality score high excluding only the module reference species, rated as good in the most upstream sampling location (Polsterlucke, close to the "Schiederweiher" ranked as Austria´s most beautiful place in 2018, https://oesterreich.orf.at/v2/stories/2943949/). Overall, the ecological status class has been scored as **high** at all the three sampling locations (Polsterlucke, Hinterstoder and Schrattentalerbrücke). Polsterlucke is classified as oligotrophic, Hinterstoder and Schrattentalerbrücke as oligo-mesotrophic (Donabaum & Krisa, 2020).

Additional information through HTS (high throughput sequencing)

+) For cyanobacteria and diatoms correspondence between microscopy and sequencing is useful to confirm microscope based identification of genera and species, and thus render the expert assessment more reproducible.

+) Additional reference species detected by sequencing might be relevant for assessment of ecological status, i.e. there were no reference species of red algae determined by the microscope, whereas the sequencing method detected two species of red algae (e.g., *Audouinella hermannii, Batrachospermum boryanum).*

+) The detection of potentially toxigenic species (i. e. *Tychonema* genotypes) might be used for early warning under conditions of increased phytobenthic growth.

7.2 Fish composition

Ecological status according to national legislative

In 2015 when the last WFD assessment was carried out, the ecological status of fish was rated as **moderate,** as no indicator species such as *Thymallus thymallus* and *Cottus Gobio* were caught. (Bayrhammer 2015)

Additional information through HTS (high throughput sequencing)

+) Using the eDNA method, not only indicator species *Salmo trutta* was detected in the highest proportion (75%) but also *Cottus Gobio* was detected in quite high proportions (12%). This information would definitely improve the estimate of the ecological status.

+) However, several non-native species, such as *Onchorynchus mykiss* (6%), *Salvelinus fontinalis (*4%), *Rutilus rutilus (*1%), *Cyprinus carpio* (1%), *Coregonus lavaretus* (0.5%) and *Perca fluviatilis* (0.5%) were detected in low proportions.

+) The molecular traces of those fish, which do not occur naturally in the Steyr were most likely detected because of fish stocking activities in the Schiederweiher, a tributary located five kilometres upstream of the sampling point used for the eDNA assessment.

+) The detection of the DNA of these non-native species shows the risk of a possible introduction into the Steyr system, possibly during flood events, which would further decrease the ecological status of fish. Thus the additional data obtained through the eDNA approach allows the identification of certain threats to the ecosystem at an earlier stage and to respond accordingly.

8 River Drome, France

France (PP6, INRAE)

8.1 Phytobenthos (Benthic diatoms)

Valentin Vasselon, Isabelle Domaizon, Marine Vautier, Frederic Rimet, Agnes Bouchez

Ecological status according to national legislative

The French standard “biological diatom index” (IBD) was drew attentions in 2000 (Prygiel and Coste, 2000), and the IBD index is now routinely applied and used to assess the ecological status of rivers at national scale in France (Table 2). For the 4 stations studied in EAW project, the IBD classifies the Drome river with a good to very good water quality.

***Table 2** : Quality classes obtained by calculating the IBD from morphology (IBD morpho) and HTS (IBD HTS) data for the four stations of the river Drome.*

CODE_STATION	IBD Morpho	IBD HTS
DROME A CHABRILLAN	16,2	14,6
Drome à charens	16,5	20
Drome A DIE 1	16,8	20
DROME A LIVRON-SUR-DROME	18	19,4

1= Very good quality
2= Good quality
3 = Average quality
4 = Poor quality
5 = Very poor quality

Additional information through HTS (high throughput sequencing)

+) For the 4 stations studied in EAW project, the IBD obtained with the sequencing data classifies the Drome river with a good to very good quality, as with the morphology data, and then a good concordance between microscopy and HTS. That can be explained by the fact that we were able to reach the species level for most of dominants reads using HTS.

+) For two stations the quality scores are different, good in morphology and very good in HTS. One of the reasons for this difference may be that the database used to assign the HTS data was not complete. Diat.barcode (Rimet et al. 2019) is an open access curated barcode library for diatoms, and it has since been updated from Diat.barcode v7 (2018) to Diat.bacode v10 (2021), and a re-analysis of the HTS data with this updated database may show a better match. Moreover, quantification and detection of taxa are basically different between both approaches (morpho vs. HTS) which might affect final diatom community structure and thus IBD scores. For quantification, one solution would be to apply the biovolume correction factor to HTS data to obtain relative proportion of reads that are more consistent with morphological counts (IBD scores presented are not corrected). For detection, the taxonomy within Diat.barcode has been updated recently according to Adl et al. 2019, maybe the person who performed morphological counting has a different taxonomic knowledge/perception, which leads to a different identification of taxa. Could be interesting to compare both taxonomy in details to evaluate where differences come from.

+) For cyanobacteria and diatoms correspondence between microscopy and sequencing is useful to confirm microscope based identification of genera and species, and thus giving the expert assessment more reproducible.

8.2 Fish composition

No results

9 River Wertach, Germany

Germany (PP10, LfU)

9.1 Phytobenthos (Benthic diatoms)

Ecological status according to national legislative

The WFD requires monitoring of macrophytes and phytobenthos (including diatoms) for the assessment of the ecological quality of rivers, which is done by the German PHYLIB assessment method. Here only the assessment based on the modul benthic diatoms is reported.

The classification of rivers from diatoms is based on the Diatom index based on the recorded species by light microscopy and the attribution of trophic weights of the found species and the proportion of reference taxa for the given water body type.

The Diatom index and revealed good or moderate ecological status at the 5 stations in river Wertach.

Table 1 : Quality classes obtained by calculating the diatom index at five stations of the river Wertach.

Wertach Station name	Mst-Nr.	Diatomeen river type	status Diatoms	Index (decimal)
Görisried	3063	D 1.2	good	2,49
Thalhofen	3067	D 1.2	moderate	3,27
Ettringen Wehr U	3074	D 4	moderate	2,68
HymnoCare Goggeleswehr	3093	D 4	moderate	2,91
oh. Wertachbrücke Augs.	213808	D 4	good	2,24

Additional information through HTS (high throughput sequencing) for the diatom index

Diatom Index with the addition of the new contribution of the HTS species was not applied because these new species haven't the relative trophic weights in the reference list. The Index requires the 95% of species determined at least on species level, and the calculation tool handles the "unknown", new detected HTS taxa like insufficient determination.

The list of non-corresponding diatom species identified from biofilm through HTS only (rbcL reference database Diat.barcode v9) from River Wertach was impressive long with 55 additional taxa (details in delivery D_T3_2_2).

With focus on those samples with strong HTS signal but less or no finding in traditional counting, a special proof by scanning electronic microscopy (SEM) was contracted (Goos 2021). In river Wertach the following species were confirmed by the SEM study in at least one of the samples: *Achnanthidium delmontii, Encyonema ventricosum, Fistulifera saprophila, Gomphonema saprophilum,* and *Gyrosigma sciotense*.

9.2 Fish composition

Germany (PP7, LfL)

Ecological status according to national legislative

In 2019 when the last WFD assessment was carried out using the assessment tool fiBS (Dußling et al. 2009), the ecological status at both sampling sites (Görisried and Türkheim) was rated as **moderate.**

Additional information through HTS (high throughput sequencing)

Görisried:

+) Using the eDNA method (VigiDNA®) *Oncorhynchus mykiss* (32.8%), *Thymallus thymallus* (33.4%) and *Rutilus rutilus* (33.8%) were detected in almost equal proportions. 7 Species (*Salmo trutta*, *Cottus gobio*, *Phoxinus phoxinus*, *Alburnoides bipunctatus*, *Squalius cephalus*, *Barbus barbus* and *Barbatula barbatula*) which were detected by traditional methods were not confirmed by the eDNA approach.

Türkheim:

+) From 11 species detected by traditional methods *Barbus barbus* (12.63%), Alburnus alburnus (4.09%), *Abramis abramis* (1.44%), *Squalius cephalus* (41.32%), *Gobio gobio* (11.49%), *Alburnoides bipunctatus* (13.63%), *Barbartula barbartula* (1.54%), Rutilus rutilus (10.09%) and *Siluris glanis* (0.94%) could be confirmed by the eDNA approach (VigiDNA®). Only *Anguilla Anguilla* and *Tinca tinca* were missing.

+) *Additionally, Perca fluviatilis (1.43%)* and *Oncorhynchus mykiss (1.40%)* were only confirmed by eDNA (VigiDNA®).

10 River Adige, Zevio, Italy

Italy (PP3, ARPAV)

10.1 Phytobenthos (Diatoms)

Giorgio Franzini, Chiara Zampieri, Federica Giacomazzi, Giampaolo Fusato, Manuela Cason

Ecological status according to national legislative

River Adige is largely used for supply of water destined for drinking (after treatment) and irrigation, power generation and as a recreation area (navigable for recreational activities). The relevant metric EQR is derived from ICMi Index (IPS20 index combined with TI index, two types of indeces based on Diatoms). The ecological quality was estimated as excellent in 2019 (Veneto-Zevio Sampling point and Pescantina Sampling point).

Additional information through HTS (high throughput sequencing)

1. The HTS can help to detect the diatom species that have escaped the LM count (biodiversity).
2. Many taxa detectable by HTS are identify at genus or family level and are unusable for the Index
3. It should be emphasized that the HTS method detected rather large diatom species such as *Didymosphenia geminata* and *Diatoma vulgaris* in discrete quantities not detected by LM. This could be an anomaly because these diatoms are easy to see under the microscope and are unlikely to have escaped the microscopist.
4. The method applied in Italy foresees to stop at the count of 400 diatom valves, this means that many taxa identified by the HTS method and present in low quantities in the sample may not have been reported in the LM lists due to the characteristics of the index. In conclusion, the contribution of information deriving from the HTS method must be introduced into the current index of Diatoms with great attention at least for biodiversity and the results must be considered indicatively.
5. We tried to convert HTS reads in number of valves, with elaboration between LM and HTS data, to apply the ICMi index with the additional HTS taxa. In every data processing attempt the indices values indicated an excellent ecological status of the river (with a slight lowering of the index value)

10.2 Fish composition

Not in use.

11 Soča River, Slovenia

Slovenia (PP5, ARSO)

11.1 Phytobenthos (benthic diatoms)

Katarina Novak, Aleksandra Krivograd Klemenčič

Ecological status according to national legislative

Phytobenthos is used as a quality element to assess the ecological status for several reasons: its sampling is simple, reacts to changes in water quality, and has a short generation time, which is why it responds to changes in the environment first. Phytobenthos is one of the biological quality elements based on which we evaluate the ecological status of watercourses. In Slovenia, only diatoms (Bacillariophyceae) are used for ecological status assessment. To assess the ecological status of watercourses based on phytobenthos, it is necessary to assess the status according to the following modules: a) modulus of trophic - trophic index (TI) with which we see mainly the impact of eutrophication and changed land use in the catchment area of the watercourse, and b) module of saprobity - based on Saprobic index (SI) assess the impact on water load with organic matter and other pollution.

Overall, the ecological status class has been scored as **high** at all three sampling sites (Spodnja Trenta, Kamno, and Solkanski jez).

Additional information through HTS (high throughput sequencing)

+) For diatoms, correspondence between microscopy and sequencing is useful for confirming microscope-based identification of genera and species and thus rendering the expert assessment more reproducible.

11.2 Fish composition

Katarina Novak, Nataša Dolinar

Ecological status according to national legislative

Fish is one of the biological quality elements based on which the ecological status of watercourses is evaluated. In Slovenia, for the time being, it is evaluated according to one module, i.e., module of general degradation. The situation according to this module is evaluated based on the Slovenian Index for the Assessment of the Ecological Status of Fish-Based Watercourses (SIFAIR). It mainly evaluates the effects of changed hydromorphological characteristics, the presence of dams, changed land use, and other pollution. The index is multimetric (consisting of various biological metrics) and is specific for each fish type. The fish type in the sample site have 8 representative/reference fish species (*Salmo marmorata*, *Cottus gobio*, *Thymallus thymallus*, *Telestes souffia*, *Phoxinus phoxinus*, *Barbus balcanicus*, *Squalius squalus*, *Barbus plebejus*).

In 2017, the ecological status of fish was assessed as **moderate**, as only *Salmo marmorata*, *Cottus gobio*, *Thymallus thymallus* were sampled as reference species (Podgornik, Ivenčnik, 2018).

Additional information through HTS (high throughput sequencing)

+) more species were detected by the eDNA method, but only two were reference species, which is less than in the traditional catch.

+) 12.5% of them were identified up to the level of the family Salmoninae, which could represent *Salmo marmorata* (6.1% in the traditional methed - catch), but their genome is difficult to identify beyond the level of the family due to crossing with *Oncorhynchus mykiss*.

+) Several non-native species, such as *Onchorynchus mykiss* (45,2%), Barbus ciscaucasicus (0,2%) were detected. The proportion of *Onchorynchus mykiss* was underestimated with eDNA method due to the fact it represent 56,9% of catch with the traditional method.

+) The molecular traces of those fish, which do not occur naturally in the Soča River could be detected because of fish farm activities (*Salvelinus*), which is upstream of the sampling sites used for the eDNA assessment.

+) The detection of the DNA of these non-native species shows the risk of a possible introduction into the Soča system, which would further decrease the ecological status of fish. Thus the additional data obtained through the eDNA approach allows the identification of certain threats to the ecosystem at an earlier stage and to respond accordingly.

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