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.T3.2.2

Report on results obtained in the five key rivers

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Contents

Introduction

Compiled from EAW meta data collection by Tina Elersek, (NIB, PP4)

All key rivers (Adige, Drome, Soča, Steyr, Wertach) are rivers with alpine influence. Almost half (45%) of river samples came from rivers with catchment area bigger than 1000 km^2, followed by medium-sized catchment area 101-1000 km^2 (36%), with 14% of river samples from rivers with catchment area below 50 km^2. During sampling campaigns, the temperature of water was ranging from 6 to 21°C, for the majority of samples (76%) in the interval 10-20°C. Conductivity was between 166 and 430 µS/cm, with a bit over half of samples (57%) in the interval 200-375 µS/cm. In our key rivers we have gathered 25 substrata samples of biofilm. Dry weight of biofilm samples was mainly in the interval below 4 g/L (59%), but surprisingly 27% of samples exhibit quite high dry weight (>12 g/L).

Trophic status

Since there is no common trophic classification of rivers, the trophic status of key rivers has been assessed by three nutrient parameters: **total phosphorus, phosphate** and **nitrate concentration**. For the analyses we used eutrophication limit boundaries for phosphorus and nitrogen concentration according to Eutrophication measures from EU Commission staff working document* (document from 2018; but data covering 2008-2015 were used). **Measured total phosphorus** concentrations corresponded to oligotrophic state in 43% of samples, and to mesotrophic state in 57% of samples (Fig. 1). The results are limited to 23 river samples from Steyr, Drome, Soča and Wertach. **Phosphate, in a form of soluble reactive form**, reached concentrations up to 0,04 mg/L in all samples, corresponding to oligotrophic state (Fig. 1). The results are limited to 16 river samples from Steyr and Wertach. Similarly, the maximum **nitrate concentrations** in all samples from key rivers were 1,2 mg/L, corresponding to oligotrophic state (Fig. 1). The results are limited to 19 river samples from Steyr, Soča and Wertach.

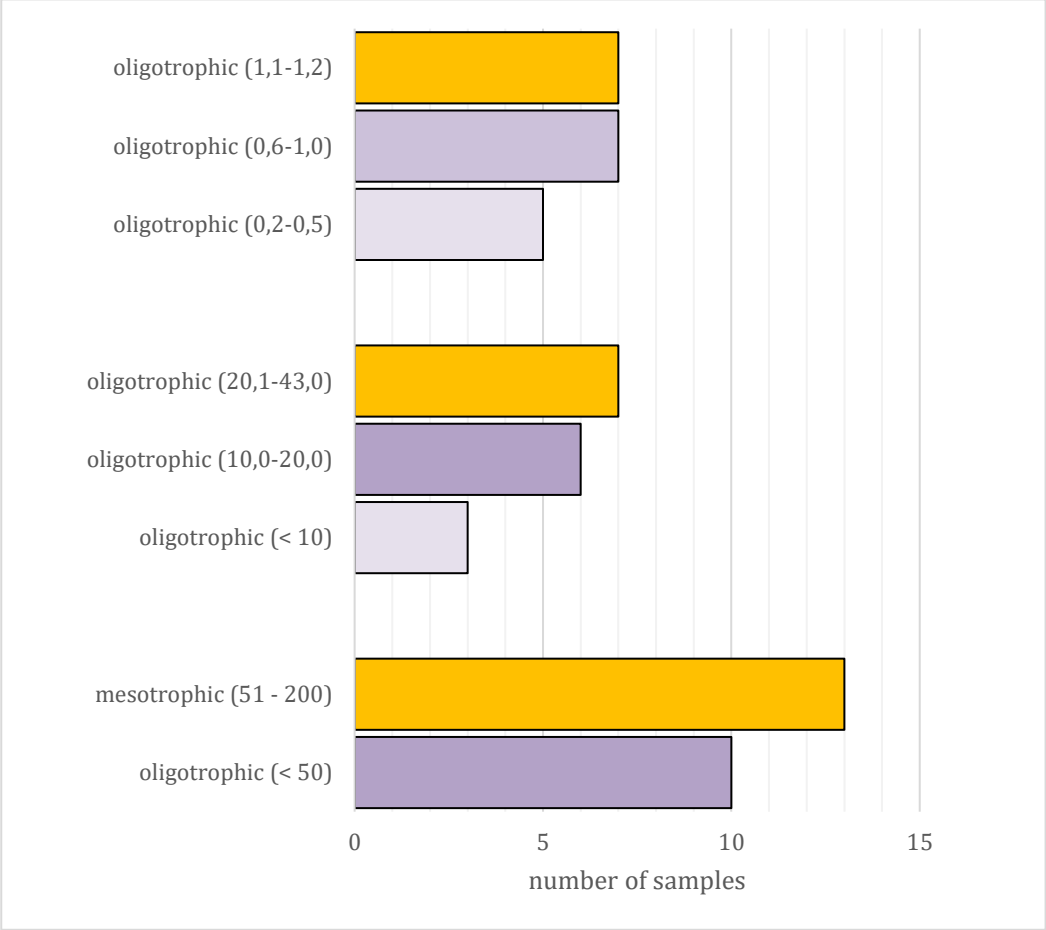

*Fig. 1. Characterisation of key river samples from biofilm (BFM) according to (i) TP - total phosphorous concentration exhibit oligotrophic and mesotrophic state, and (ii) PO4 - phosphate and (iii) NO3 - nitrate concentration, exhibit oligotrophic state, according to the EU commission report *. Styer, Drome and Soča exhibit lower values, while Wertach showed higher values (dark orange color).*

1 River Steyr, Austria

1.1 Phytobenthos (incl. cyanobacteria)

Austria (PP2, LFUI)

Rainer Kurmayer, Hans Rund, Josef Wanzenböck

Phytobenthos has proven to be an indicator for ecological quality status in rivers. In Austria all phytobenthic algae groups, including Cyanobacteria, are used as biological quality elements. Exempt from this are only Charophyceae who, by tradition, are recorded within the scope of the macrophyte method.

Sampling according to national legislative

River Steyr (Upper Austria, 68 km in length) is a small river originating in totes Gebirge (850 m a SL) and draining into the Enns at Steyr city (290 m a SL). The discharge at middle water level is 36.4 m^3/sec (Pegel Pergern) representing a low order stream. The river Steyr and its river basin is used for hydropower

generation. It is also known as a rather distinguished recreation site (e.g. in Oct 2018 the reservoir Schiederweiher in Hinterstoder has been designated by public voting as the most beautiful place in Upper Austria). The River Steyr already has served as pilot site for the alpine space project SPARE (Strategic Planning of Activities in River Ecosystems) focusing on river management (2015-2018). Sampling was performed from GZÜV sampling sites, i.e, Polsterlucke (P1), Hinterstoder (P2), Schrattentalerbrücke (P3) at 3 September 2019.

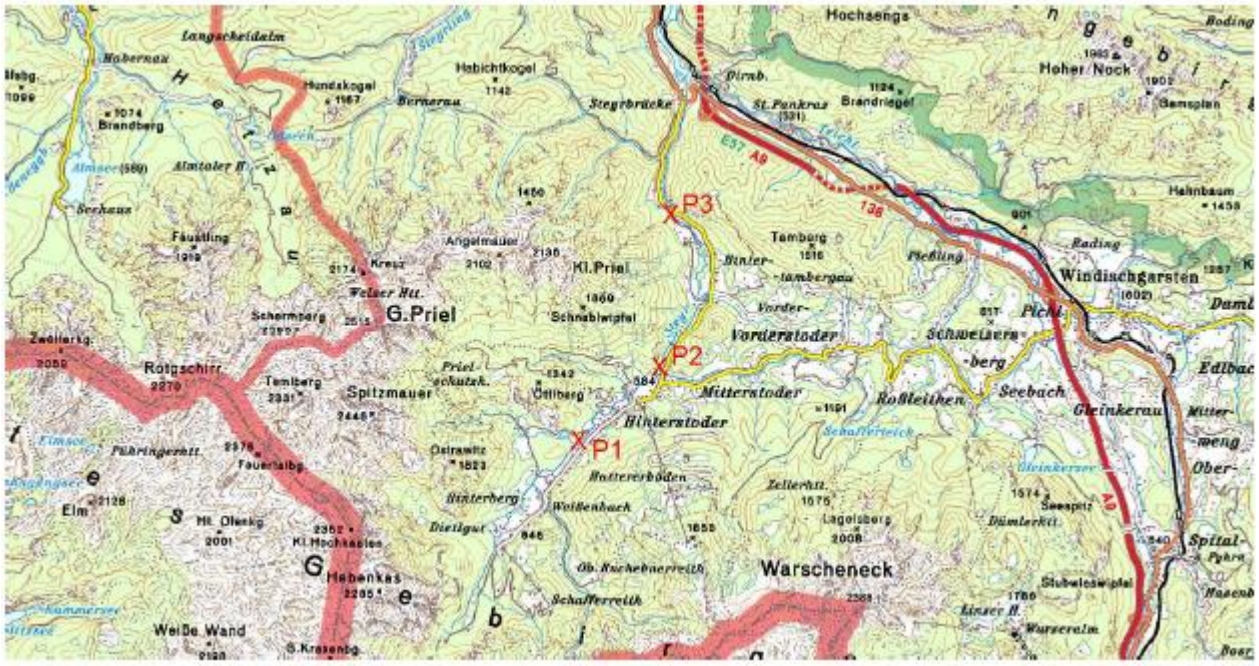

Fig. 1.1. Sampling sites for River Steyr (Upper Austria), P1, Polsterlucke; P2, Hinterstoder; P3, Schrattentalerbrücke

In general sampling was performed according to the national legislative for phytobenthic sampling (Pfister & Pipp 2015) performed by DWS HydroÖkologie GmbH (Vienna, Austria). To estimate the surface coverage of phytobenthic algae an "Aquascope" was used, which allows for underwater inspection of macroscopic and microscopic algae growing in mixed populations. Five stones were selected by wading into the water and algae growing at the stone surface were brushed off into a tray. Both soft algae and diatoms were sampled using formaldehyde fixation (2%).

In parallel chemical-physical parameters were determined using multiprobes, while for chemical analysis water from 0.2 m depth was collected. Total phosphorus (TP) ranged from 4 µg/L (P2) to 6 µg/L (P1, P3). The reactive nitrogen (NO3-N) concentration was 0.4 mg/L at all three sampling sites indicating oligotrophic conditions.

In parallel to sampling for microscopy, for DNA extraction from the same stones aliquots were preserved using 80% Ethanol as described in protocol (DT1.1.2. -2, Lake biofilms sampling protocol).

Finally aliquots were scratched directly onto pre-weighed GF/C Filters and the dry-weight was determined from the difference in dried filter (105°C, 24 h) weight before and after filtration. Aliquots without drying but stored at -20°C were then used for cyanotoxin extraction (protocol: Cyanotoxins analyses in lake and biofilm samples).

Results on cyanotoxins concentrations

No cyanotoxins were detected either on Polsterlucke (P1), Hinterstoder (P2), Schrattentalerbrücke (P3).

DNA extraction and sequencing

DNA was extracted using the Machery and Nagel NucleoSpin® Soil kitDNeasy® following the WP1 protocol (DT1.1.2. -7, DNA extraction biofilms)

From sample DNA extracts 16S rDNA (V3-V4 region) has been amplified using primers 341Fmod CCTAYGGGRBGCASCAG and 806Rmod GGACTACNNGGGTATCTAAT under the following conditions: 95°C (5 min), 28 cycles including 95°C (30 sec), 55°C (30 sec), 72°C (30 sec), and final 72°C (5 min). For 18S rDNA (V4 region) the primers V4F-18S_ILL and CCAGCASCYGCGGTAATTCC and V4R-18S_ILL ACTTTCGTTCTTGATYRATGA were applied using the cycling conditions from above. One technical replicate was sequenced (DT1.1.2. -10 Library prep 16S marker gene; DT1.1.2. -11, Library prep 18S marker gene).

PCR amplification and library preparation of purified PCR products for rbcL was performed according to WP1 protocol (DT1.1.2. -9, Library prep RbcL marker gene). Bridge amplification and sequencing by synthesis were performed according to Miseq standard conditions.

Bioinformatic processing

The raw sequence data were processed using the package Divisive Amplicon Denoising Algorithm 2 (DADA2), (DT1.1.3. - 1 BioinfRbcL, Bioinformatics treatment RbCL marker gene, DT1.1.3. - 3 Bioinf18S, Bioinformatics treatment 18S marker gene, DT1.1.3. -2 Bioinf16S, Bioinformatics treatment 16S marker gene).

Sequences were clustered into ASVs (no dissimilarity threshold) and assigned to the SILVA SSU reference database (or PR2 database?) for taxonomic classification. For rbcL gene assignment to diatom taxa the curated database R-Syst::diatom (Rimet et al. 2016) was used (INRA).

Comparison with traditional microscopy

All microscopical taxa lists have been standardized using the established WFD (EU project WISER) taxa codes, i.e. the VALID code system for diatoms in phytobenthos (LfU) and the REBECCA code for non-diatoms (soft algae) An Excel Access database for all microscopical taxa and the VALID codes assigned has been prepared (LfU, FEM, LFUI).

In traditional phytobenthic assessments, diatoms and non-diatoms (soft algae) are evaluated at a ratio of 1:1 summing up to 200%. Microscopical countings were performed according to the national legislative by DWS HydroÖkologie GmbH (Vienna, Austria).

Results on comparison between traditional microscopy and HTS

Macroscopic inspection revealed thin algal growth (growth depth of 1 mm) on the stone surface, with a percentage of coverage 69, 61, 56 % for sites P1, P2,P3, respectively.

As inferred from microscopy **within soft algae** algal groups comprised Chrysophyceae (63%), cyanobacteria (19%), Chlorophyta (17) and red algae (< 1%). The most abundant taxa included *Phaeodermatium rivulare, Gongrosira incrustans, Hydrurus foetidus, Plectonema tomasinianum, Chamaesiphon geitleri, Pleurocapsa aurantiaca. P. rivulare, H. foetidus, P. tomasinianum, C. geitleri*.

According to the Austrian phytobenthos trophic indication system (Pfister et al. 2016) *P. rivulare*, *H. foetidus*, *P. tomasianum*, *C. geitleri* are all indicative of oligotrophic conditions. In contrast G. incrustans and *G. debaryana* are indicative of more eutrophic conditions. Finally *Chlorogloea microcystoides* is indicative of eutrophic conditions but occurred in low abundance at sampling site P3 only.

In general taxonomic composition between both methods corresponded on genus level, i.e. the filamentous non-heterocystous genera *Leptolyngbya* and *Pseudanabaena* (Order Synechococcales) as well as *Phormidium* were represented. In addition the benthic genera *Chamaesiphon* and *Pleurocapsa* were detected by both methods. Notably the genus *Tychonema* as well as the heterocyst-forming genus *Calothrix* were not recorded during microscopical analysis but detected through 16S rDNA HTS. Previous unknown cyanobacteria included (i) the coccale cyanobacterium *Aliterella,* which has been described as a marine deep water or benthic species (Rigonato et al. 2016) and (ii) the thin filamentous cyanobacterium genus *Phormidesmis* described from stones in oligotrophic glacial streams or subaerophytic from cold wet rocks (Raabova et al. 2019).

Within diatoms *Achnanthidium lineare, Achnanthidium pyrenaicum, Achnanthidium affine, Gomphonema angustivalva* contributed >80% at all three sampling stations. The majority of these taxa were representative

of the so-called low profile guild (Passy 2007), while members of the high profile guild (chain-forming diatoms) and motile taxa were practically absent.

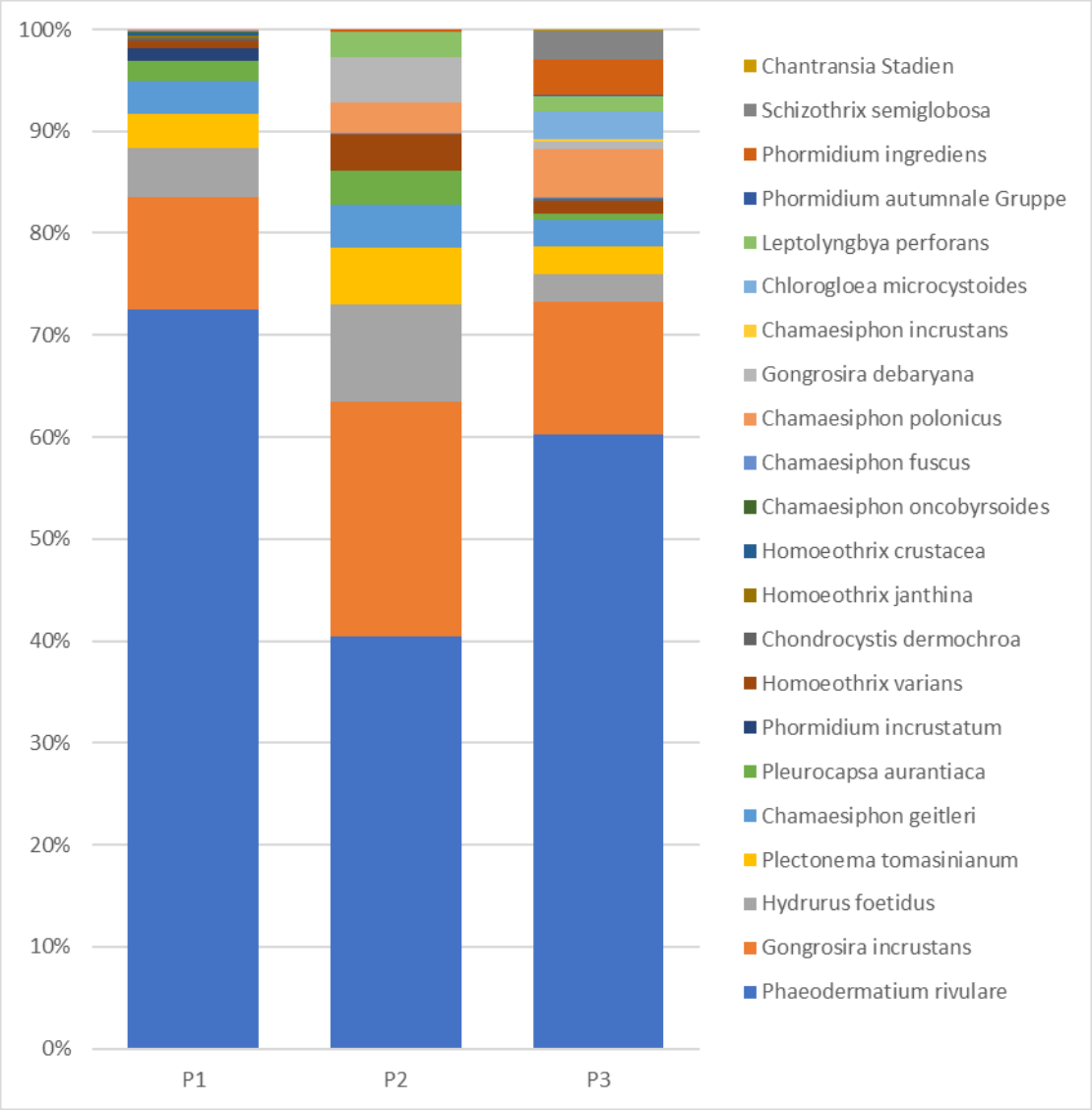

Fig. 1.2. Relative abundance of cyanobacteria and other algal groups at three riverine sampling sites from River Steyr, P1, Polsterlucke; P2, Hinterstoder; P3, Schrattentalerbrücke as revealed from microscopical counting (for location of sites see Fig. 1.1).

As for cyanobacteria, taxonomic composition between both methods corresponded on genus level, i.e. the genera *Achnanthidium, Gomphonema, Encyonopsis, Amphora, Cocconeis, Encyonema, Navicula* were included. In addition a relatively high number, 48 diatom species were detected by both methods, i.e. sequencing (18S: n=13, rbcL: n=34) and microscopy.

Vice versa 24 morphospecies which were differentiated in the microscope were not recorded either via 18S rDNA or rbcL sequencing. Those taxa included morphospecies of the genera A*chnanthidium, Amphora, Cocconeis , Cymbella, Diatoma, Encyonema , Fragilaria, Geissleria , Gomphonema , Navicula , Reimeria.* Interestingly for this key river site, sequencing (either through rbcL or 18S rDNA) did not reveal additional taxa that were not recorded through the microscope. This is in contrast to the key lake Mondsee, where among biofilm samples 89 diatom morphospecies were not detected in the microscope but through sequencing.

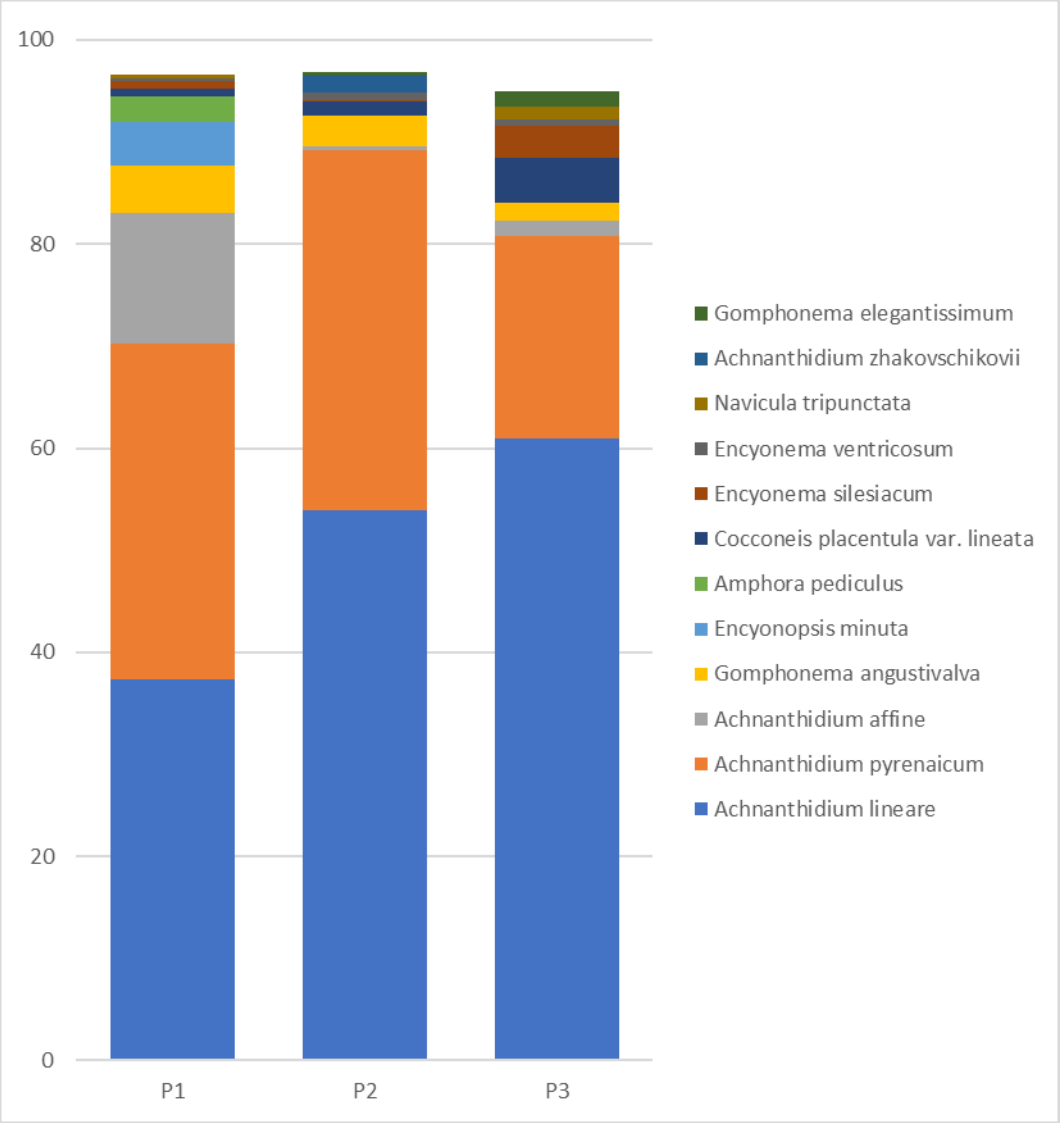

Fig. 1.3. Relative abundance of diatoms (> 2% of total counts) at three riverine sampling sites from River Steyr, P1, Polsterlucke; P2, Hinterstoder; P3, Schrattentalerbrücke as revealed from microscopical counting (for location of sites see Fig. 1.1).

Conclusion on results obtained for phytobenthos (cyanobacteria & diatoms)

Relevant information derived from sequencing includes the following:

(i) For cyanobacteria correspondence between microscopy and 16S rDNA sequencing is useful to confirm microscope based identification of genera.

(ii) Alternatively previously unknown cyanobacteria have been detected that might require further study (genera *Aliterella, Phormidesmis*)

(iii) The 16S rDNA sequencing information can be useful to infer the toxigenic potential of the respective biofilm community, e.g. at site P2 & P3 the *Tychonema* genotype Seq No34 has been detected which has been linked to anatoxin-a production in the plankton previously (L. Como, L. Garda,L. Iseo, L. Ledro, L. Maggiore, Staffelsee Nord).

(iv) For diatoms correspondence between microscopy and rbcL or 18S rDNA sequencing is considered useful to confirm microscope based identification of genera, e.g. for invasive species (*A. delmontii*).

(v) Since no additional diatom taxa were recorded through sequencing (neither rbcL nor 18S rDNA) the sampling depth of microscopical analysis is considered high

1.2 Fish composition, River Steyr

Sampling

The sampling on the River Steyr took place 02.09.2019 at sampling site Schrattentaler Brücke (47.744605, 14.167110) according to the Eco-AlpsWater protocol D.T1.3.1-4 - Lake and river eDNA fish sample collection from the field for downstream molecular analysis. Two different eDNA approaches (both of them are described in detail in the protocol) have been carried out and compared.

Figure 1: Sampling site river Steyr

VigiDNA®:

Standard sampling: 30 liters of water were taken main current using a peristaltic pump system and filters directly through VigiDNA® 0.45 µm filter cartridges. After filtration, the cartridges were filled with a preservation buffer and stored in the fridge until DNA extraction according to Eco-Alpswater protocol D.T1.3.1-4 Lake and river eDNA fish sampling. In the meantime, however, we would no longer recommend storing the samples in the refrigerator due to difficulties, especially with regard to DNA extraction. Therefore, it is advised to store the samples at room temperature until extraction.

GFC:

Additional sampling: For each sample, 5 liters of water were collected using a DNA-free container. In total, 9 samples (3 at each river bank and 3 in the main current) were taken along a 100 meter stretch. Back in the laboratory, the samples were filtered through glass fiber filter discs (GFC) 1.2 µm using a vertical filtration device. After filtration, the filters were stored frozen at -20° until DNA extraction.

DNA extraction and sequencing

For the fish eDNA extraction from VigiDNA® cartridges a combination of the Macherey-Nagel NucleoSpin® and the DNeasy Soil Kit® was used according to the Eco-Alpswater protocol D.T1.3.1-8.2 - Fish DNA extraction from VigiDNA® cartridges. For the fish eDNA extraction from GFC filters, the DNeasy Power Water kit (Qiagen) was used, following the manufacturer's protocol.

The PCR amplification as well as the library preparation was done by AGES (Austrian Agency for Health and Food Safety) according to the the Eco-Alpswater protocol D.T1.3.1-12 - Library preparation 12S. For the sequencing, MiFish-U primers (forward: 5`- GTCGGTAAAACTCGTGCCAGC-3`, reverse: 5`- CATAGTGGGGTAT-

CTAATCCCAGTTTG-3`, Miya et al. 2015) were used and for each sample. For each VigiDNA® sample nine replicates were performed, for the GFC filters only one.

Bioinformatic processing

Raw sequencing data were analyzed at the Research Department for Limnology, Mondsee. For the bioinformatics analysis, the qiime2 pipeline (Bolyen et al. 2019) was used. This pipeline was originally designed to work on microbiome data. However, previous test showed, that the taxonomic assignment of the obitools3 pipeline, which was used by most partners in the EAW project, and the taxonomic assignment of the qiime2 pipeline delivered comparable results regarding the taxonomic assignment of fish in eDNA samples. Due to easier handling of the bioinformatics processes and a slightly finer taxonomic resolution, the German and Austrian project partners used the qiime2 approach.

Comparison with traditional fish monitoring

The taxonomic inventories obtained from the bioinformatic analysis were then compared to the dataset obtained from the traditional fish sampling at River Steyr, which was carried out in 2015. The traditional method consisted of electrofishing along a 200 meter stretch.

Results on comparison between traditional monitoring and HTS

VigiDNA®:

For the VigiDNA® sample, 9 replicates were sequenced. For the analysis, the average number of reads per species (occurring in the 9 replicates) was used. In total 4 fish species (Table 1, Figure 2 - B) were detected during the EAW sampling campaign (2019) and the traditional sampling campaign (2015). 1 fish species (25%) was detected by both methods, 0 fish species were identified only by the traditional methods (electrofishing) and 3 fish species (75%) were detected only with the HTS approach.

GFC:
No replicates were used in this approach, the number of reads for each species in the 9 samples, was summed up. In total 8 fish species (Table 1, Figure 2 - A) were detected during the EAW sampling campaign (2019) and the traditional sampling campaign (2015). 1 fish species (12,5%) were detected by both methods, 0 fish species were identified only by the traditional methods (electrofishing) and 7 fish species (87,5%) were detected only with the HTS approach. Not only were all species, detected with the VigiDNA® filters and the traditional methods, detected with the GFC filters, but also 4 additional species.

Table 1: Comparison of fish taxa detected with traditional and eDNA (VigiDNA® and GFC) assessment methods at sampling site Schrattentaler Brücke at River Steyr. The numbers in the molecular method column shows the total number of reads for each species and method. The traditional methods columns show the number of individuals caught by electrofishing

Common name	Scientific name	Molecular methods			Traditional methods
		VigiDNA®	GFC	Total	Electrofishing
Brown trout	*Salmo trutta*	17609	1734667	1752276	12
Bullhead	*Cottus gobio*	10674	292737	303411	0
Rainbow trout	*Onchorynchus mykiss*	7176	136129	143305	0
Brook trout	*Salvelinus fontinalis*	9123	96622	105745	0
Roach	*Rutilus rutilus*	0	18727	18727	0
Common carp	*Cyprinus carpio*	0	15431	15431	0
European whitefish	*Coregonus lavaretus*	0	10586	10586	0
Perch	*Perca fluviatilis*	0	10510	10510	0

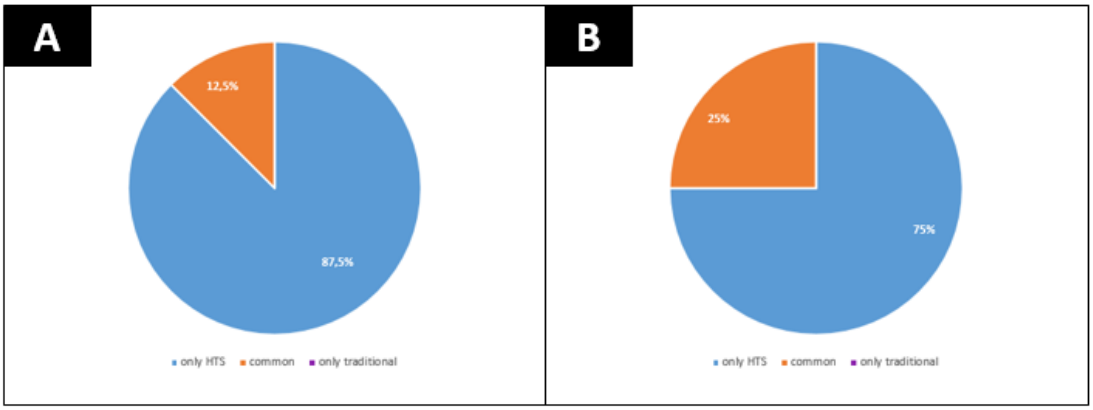

Figure 2 shows the percentage of species detected by molecular (GFC = A and VigiDNA® = B) and traditional assessment methods (electrofishing) at the River Steyr.

Conclusion on results obtained for fish

eDNA metabarcoding for fish is a valuable tool to quickly assess the species composition of aquatic ecosystems. Due to the low number of different species, detected by electrofishing (only 1), both eDNA methods outperformed the traditional approach. The fact that some fish species could only be detected with GFC but not with VigiDNA® filters could be because the extraction of the VigiDNA® cartridges was not optimal due to incorrect storage conditions (fridge) and bacterial growth in the buffer which led to DNA degradation. However, both eDNA approaches were able to detect species that were not caught during the traditional sampling event, including several non-native species (VigiDNA® = 3, GFC = 7). 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.

2 River Drôme, France

2.1 Phytobenthos (benthic diatoms)

France (PP6, INRAE)

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

Phytobenthos has proven to be an indicator for ecological quality status in rivers. In France, diatoms algal groups are used as biological quality elements.

Sampling according to national legislative

River Drôme is a river originating in “La Bâtie des Fonds” (1262 m) and draining into the Rhône after Livron-sur-Drôme city (91 m). The discharge at middle water level is 20 m^3/sec representing a low order stream. The River Drôme already served as pilot site for the alpine space project SPARE (Strategic Planning of Activities in River Ecosystems) focusing on river management (2015-2018). Sampling was performed from four DREALs sampling sites: “Drôme à Livron-sur-Drôme”, “Drôme à Chabrillan”, Drôme à Die” and “Drôme à Charens” the 11 July 2018.

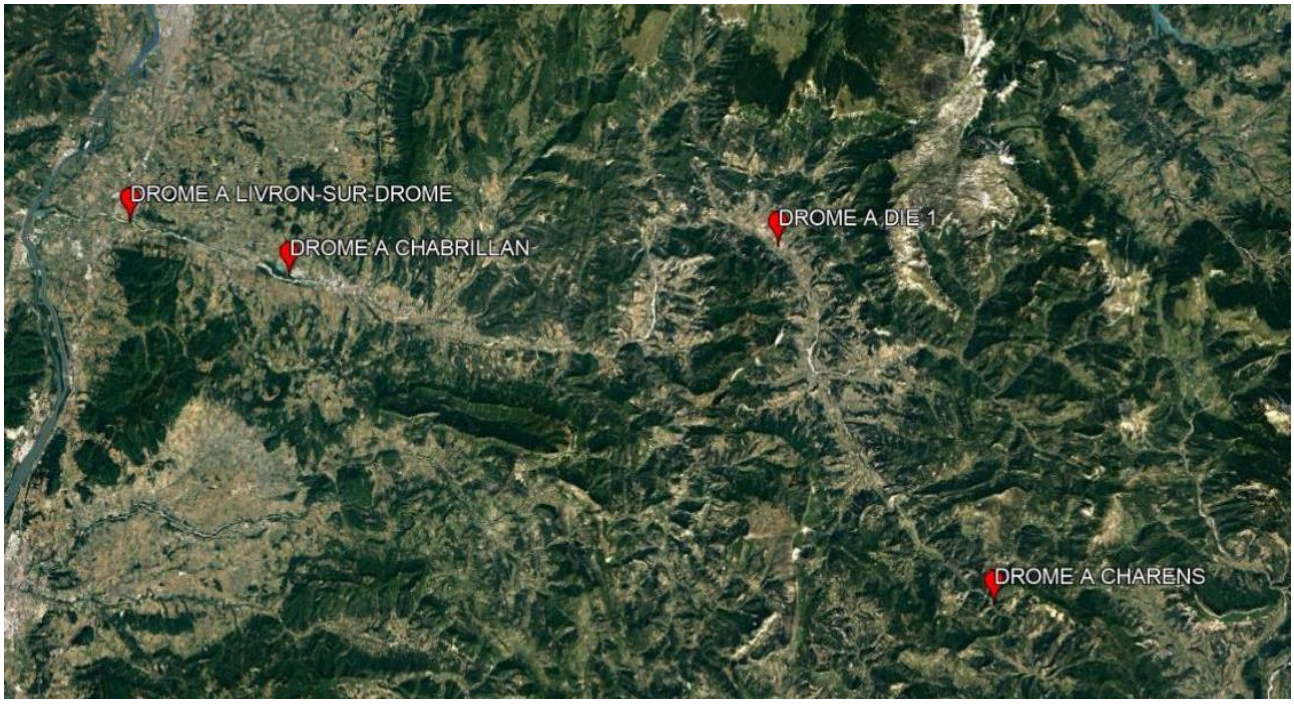

Fig. 2.1. Sampling sites for River Drôme.

For each site, 5 stones were selected along the shoreline representing an area of 50-100 cm^2. Samples were brushed off from stones from a representative surface area using a clean tray.

From the same stones aliquots, biofilms were preserved in 80% Ethanol as described in protocol (D.T1.3.1-3, River biofilm sampling protocol) in two different tubes, and diatoms were identified either by microscopic analysis or by eDNA analysis.

The samples from the 4 sites were analyzed by both HTS and by microscopy.

DNA extraction and sequencing

DNA was extracted using the Macherey and Nagel NucleoSpin® Soil kitDNeasy® following the protocol defined in WP1 (D.T1.3.1-7, DNA extraction biofilms).

PCR amplification and library preparation of purified PCR products for rbcL (barcode selected for the analysis of diatoms diversity) was performed according to WP1 protocol (DT1.1.2. -9, Library prep RbcL marker gene). Bridge amplification and sequencing by synthesis were performed according to Miseq standard conditions.

Bioinformatic processing

The raw sequence data were processed using the package Divisive Amplicon Denoising Algorithm 2 (DADA2), (D.T1.3.2-1 BioinfRbcL, Bioinformatics treatment rbcL marker gene).

Sequences were clustered into ASVs (no dissimilarity threshold) and assignment to diatom taxa was performed using the curated database Diat.barcode v7 (Rimet et al. 2019).

Comparison with traditional microscopy

All microscopical taxa lists have been standardized using the established WFD (EU project WISER) taxa codes, i.e. the VALID code system for diatoms in phytobenthos (LfU) and the REBECCA code for non-diatoms (soft algae). An Excel Access database for all microscopical taxa and the VALID codes assigned has been prepared (LfU, FEM, LFUI).

Results on comparison between traditional microscopy and HTS (Diatoms).

Table 2.1. Comparison of diatoms taxa at genus level for river Drôme detected using the two different methods (microscopical analysis vs sequence analysis) or detected only by one method

Common	Only µscopy	Only HTS
Achnanthidium	Psammothidium	Caloneis
Amphora		Cocconeis
Cymbella		Melosira
Diatoma		Navicula
Encyonema		Reimeria
Fistulifera		Surirella
Fragilaria		
Gomphonema		
Mayamaea		
Nitzschia		
Ulnaria		

Eleven diatoms genus were detected using both methods (61% of shared genus). Six diatoms genus were found through metabarcoding, but were not detected under the microscope, and only one diatoms genus was not identified by metabarcoding, but found under the microscope (Table 2.1).

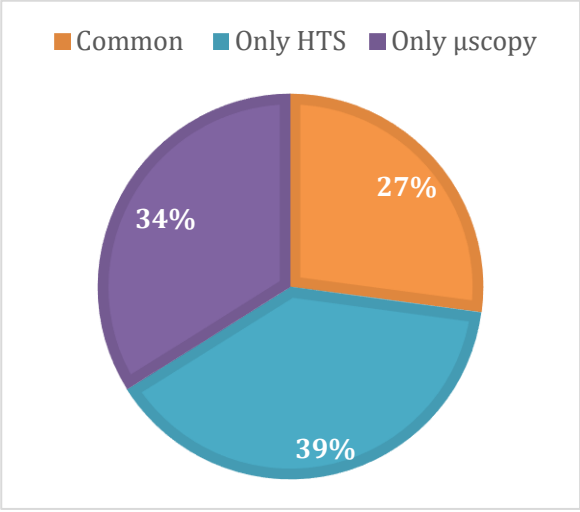

Fig. 2.2. Mean percentage of diatoms species identified by HTS and microscopy (common) (14), only microscopy (only µscopy) (20), or only by HTS (only HTS) (25), for Drôme samples.

When looking at the taxonomic assignment at the species level, the correspondence between the two methods is lower, with only 14 diatom species in common, representing 27% of all species identified by both

methods (Fig. 2.2). 20 species were identified only by microscopy, and 25 species only by HTS. The correspondence between the methods is therefore weaker at the species level than at the genus level.

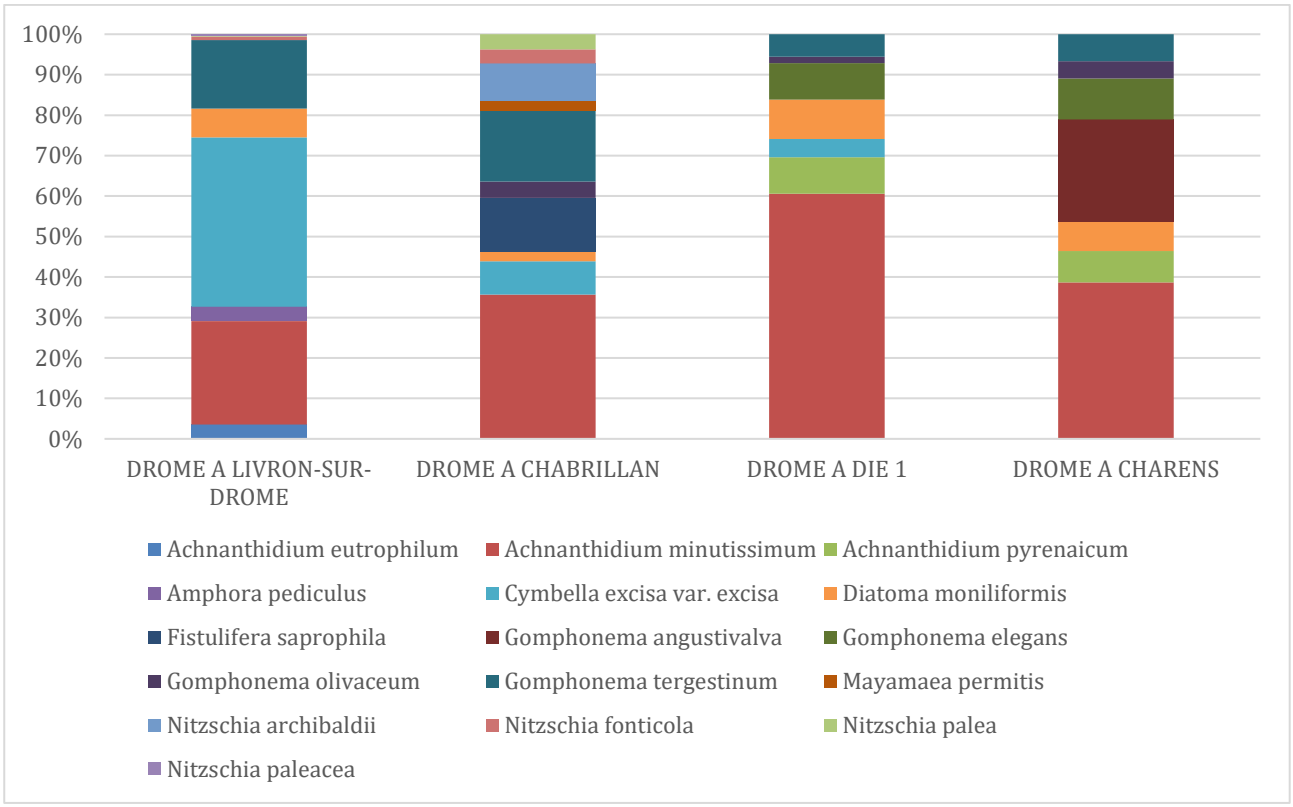

Fig.2.3. Relative abundance of diatoms (> 2% of total counts) at four riverine sampling sites from River Drôme, as revealed from microscopical counting (for location of sites see Fig. 2.1).

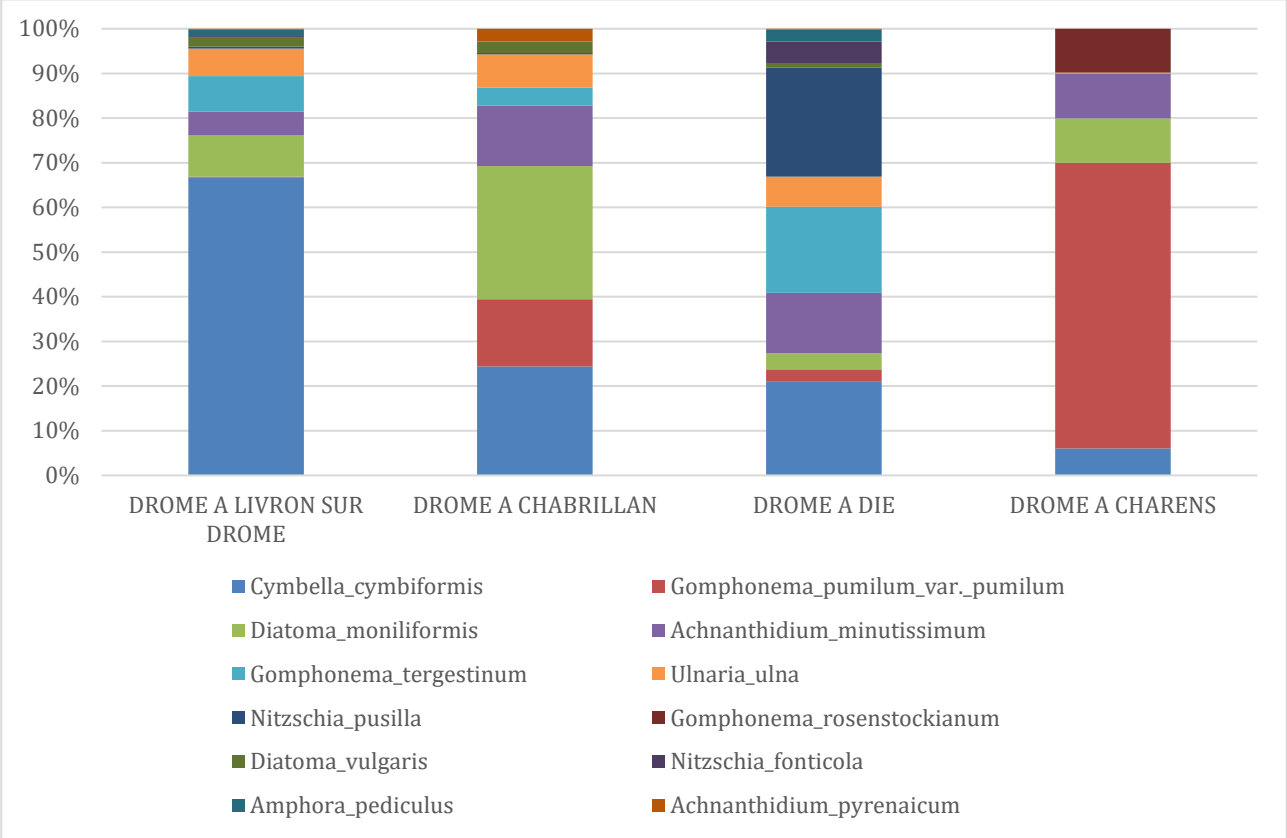

Fig. 2.4. Relative abundance of diatoms reads (> 2% of total reads) at four riverine sampling sites from River Drôme, as revealed from HTS sequencing (for location of sites see Fig. 2.1).

Dominant species were identified by both methods. However, some species were not identified by metabarcoding. In particular:

Cymbella excisa, is a species identified in microscopy in all the samples and even counted as dominant in Livron/Drome . *C. excisa* is barcoded in Diat.barcode. However, when looking at Diat.barcode database, there is one sequence identified as *C. cymbiformis,* which is in the phylogenetic clade of *C. excisa*. This is a mistake, and therefore for the next version of Diat.barcode, the taxonomic name of this sequence will be modified into *C. excisa*.

Gomphonema tergestinum was determined in microscopy but not in DNA. However, *G. rosenstokianum* is a morphologically sister species and was identified in DNA.

Several *Achnanthidium* species were identified in microscopy (*A. eutrophilum, A. lineare*). They belong to the *A. minutissimum sensu lato* species complex and were identified as *A. minutissimum* with Diat.barcode.

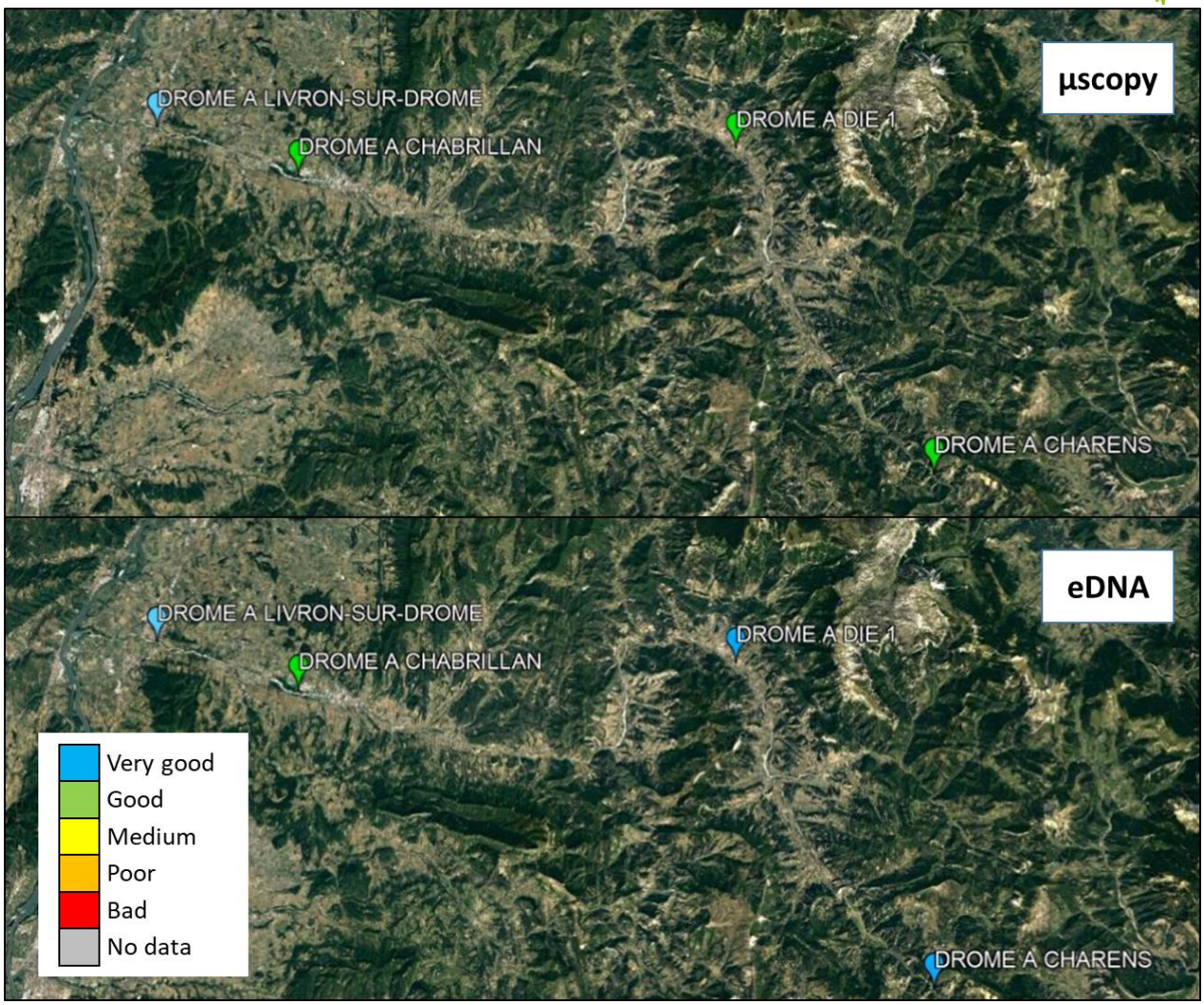

Fig. 2.5. Map of biological water quality indices calculated from HTS data (eDNA) or morphological data (µscopy), for the four river Drôme samples.

Even though only 27% of the diatom species are shared by both approaches, the calculation of the water quality indices gives similar results, although two sites score better with the eDNA approach compared to the morphological approach (Fig. 2.5).

Conclusion on results obtained for diatoms

Relevant information derived from sequencing includes the following:

(i) Good match between microscopy and HTS for assignment to genus level
(ii) Low match between microscopy and HTS at the species level, but the dominant species were identified by both methods
(iii) The diatom data in HTS allow the calculation of water quality indices, which give results close to those obtained with microscopy countings.
(iv) More work needs to be done to harmonize the traditional and HTS approaches, but the calculation of indices is possible even without a perfect match between the two approaches at species level.

2.2 Fish composition, River Drome

Not available

3 River Wertach, Germany

3.1 Phytobenthos (benthic diatoms)

Germany (PP10, LfU))

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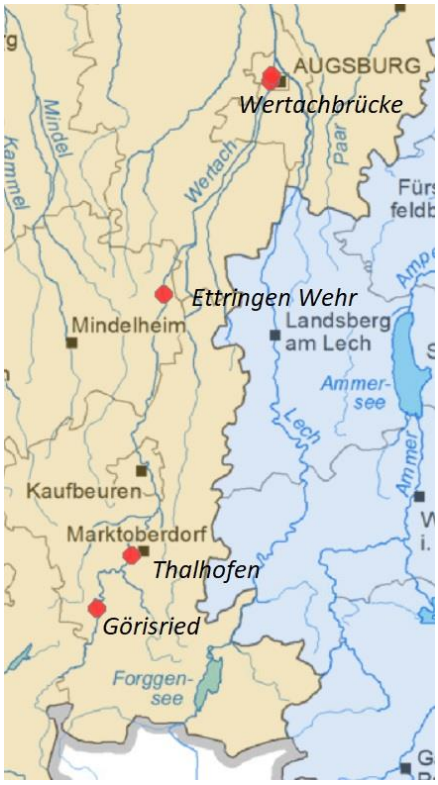
General introduction

Study site river Wertach is originating in the northern Alps near the border between Austria and Germany, and is then running in roughly north direction through Bavaria towards the Danube River. Wertach has been a typical pre-Alpine river with high sediment load. The seasonal discharge regime is shaped by snow melt in spring. Drainage area of river Wertach comprises 1441km2 and total length is 137 km.
In the period 2013-2017 the annual mean of discharge was 16.4m3/s, with distinct maxima up to 190. An additional description of the river Wertach is available by the Alpine Space project HYMOCARES (2016-2019).



Sampling

The long-term monitoring program of river Wertach is run by the regional water management administrations (WWA Kempten; WWA Donauwoerth).Five different water bodies are assigned to river Wertach by the Bavarian Environment Agency. Parameters such as nutrients and solid matter data are monitored monthly at the main station “Ettringen Wehr Unterwasser”, and at several further stations in those years with monitoring according to the EU-WFD.
Independently, biofilm (Diatoms) samples were collected in 5 stations (Görrisried, Thalhofen Pegel, Ettringen Wehr, Wertachbrücke, HymoCare station “Goggle-Wehr”) at 30 August and 1 September 2019 (map in Fig. 3.1, Fig. 3.2) during low water period according to Deliverable D.T.1.1.2-3 River biofilms sampling. *In contrast to other project partners, we sampled in parallel three nearby zonse each with 5 stones of one regular station to get information about the spatial representativity of a selection of 5 stones.*

Fig 3.1: Map of river Wertach (blue line) with red dots marking the EAW sample stations. Most southern station Görrisried is about 79km apart from stations in city Augsburg. The river runs almost in parallel to the larger Alpine river Lech to which Wertach confluences.

Diatom samples are taken according to the German method PHYLIB, which is in accordance with Deliverable D.T.1.1.2-3 River biofilms sampling. In addition to the first regular sampling per station (5 stones), LfU added two replicates (each with further 5 stones) in order to study the spatial heterogeneity of the biofilms at each station.

For the assessment of ecological status, using diatom communities, the Multimeter Intercalibration Index (PHYLIB 5.3.0 (18.02.2016) is applied by using the PHLIB software version 5.3.0 (18.02.2016).The assessment is based on the reference species index and a trophic index. The Identification of diatoms is at species or intraspecific -level.
For each site, 5 stones were selected along the shoreline. Samples were brushed off from stones from a representative area of 50-100 cm^2 using a clean tray.
From the same stones aliquots, biofilms were preserved in 80% Ethanol as described in protocol (D.T1.3.1-3, River biofilm sampling protocol) in two different tubes, and diatoms were identified both by microscopic analysis and by eDNA analysis.

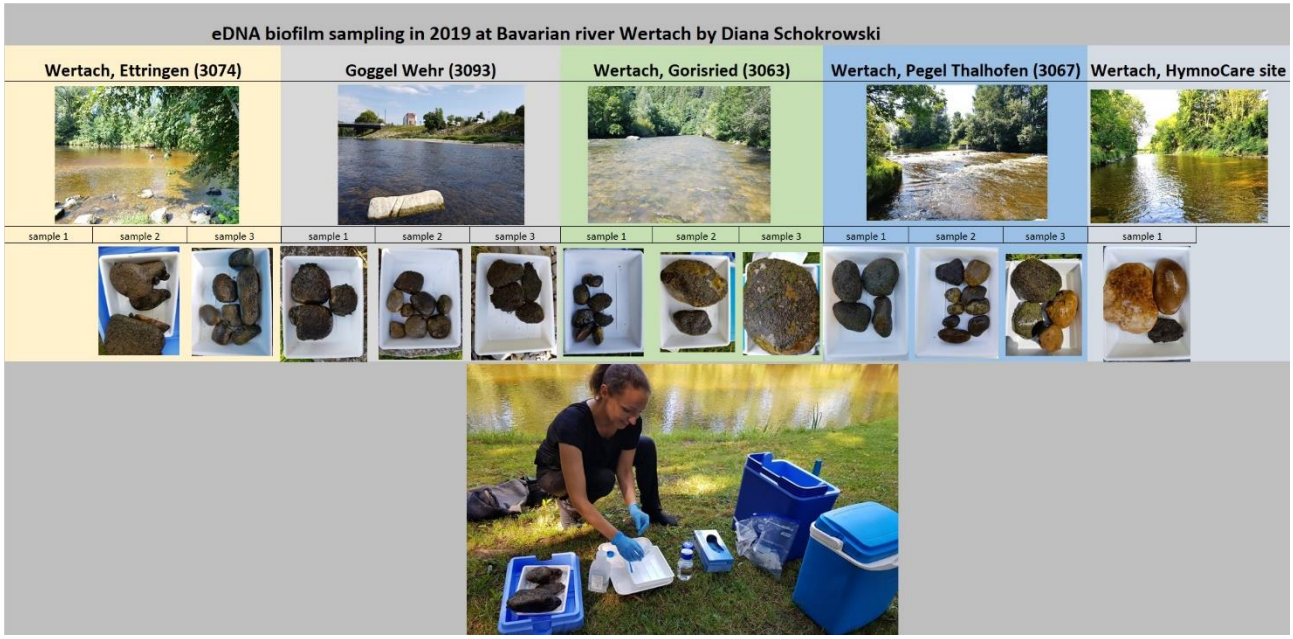


Fig. 3.2: Illustration of the sample sites at river Wertach, the collected stones and the in-situ sample processing by an external service, D. Schorkowski.

Rules to define ecological classes and reference conditions

The WFD requires monitoring of diatoms for the assessment of the ecological quality of rivers.
The classification of rivers with diatoms is based on the recorded species and the attribution of trophic weights of the found species in the German PHYLIB diatom index. In Germany for complete assessment with PHYLIB also macrophytes and other phytobenthos are sampled and assessed too, but this was not realised in the EAW sampling in year 2019 and other biological groups than diatoms are not reported here.
The reference method is reported in Schaumburg et al. (2012; PHYLIB - Assessment Procedure for Macrophytes and Phytobenthos - LfU Bayern).

Sampling and Results on cyanotoxins concentrations

No samples for cyanotxins were taken at river Wertach.

DNA extraction and sequencing

DNA was extracted using the Machery and Nagel NucleoSpin® Soil kitDNeasy® following the WP1 protocol (DT1.1.2. -7, DNA extraction biofilms) by the Italian project partner FEM.
From sample DNA extracts 16S rDNA (V3-V4 region) has been amplified using primers 341Fmod CCTAYGGGRBGCASCAG and 806Rmod GGACTACNNGGGTATCTAAT under the following conditions: 95°C (5

min), 28 cycles including 95°C (30 sec), 55°C (30 sec), 72°C (30 sec), and final 72°C (5 min). For 18S rDNA (V4 region) the primers V4F-18S_ILL and CCAGCASCYGCGGTAATTCC and V4R-18S_ILL ACTTTCGTTCTTGATYRATGA were applied using the cycling conditions from above. One technical replicate was sequenced (DT1.1.2. -10 Library prep 16S marker gene; DT1.1.2. -11, Library prep 18S marker gene).
PCR amplification and library preparation of purified PCR products for rbcL was performed according to WP1 protocol (DT1.1.2. -9, Library prep RbcL marker gene). Bridge amplification and sequencing by synthesis were performed according to Miseq standard conditions.

Bioinformatic processing

The raw sequence data were processed using the package Divisive Amplicon Denoising Algorithm 2 (DADA2, see (Protocols DT1.1.3. - 3 Bioinf18S, Bioinformatics treatment 18S marker gene; DT1.1.3. -2 Bioinf16S, Bioinformatics treatment 16S marker gene, D.T1.3.2-1 BioinfRbcL, Bioinformatics treatment rbcL marker gene). Sequences were assigned using the SILVA SSU reference database (bacteria/cyanobacteria) and the PR2 database (protists/microalgae). Sequences were clustered into ASVs (no dissimilarity threshold) and assignment to diatom taxa was performed using the curated database Diat.barcode v7 (Rimet et al. 2019).

Elaboration of traditional microscopy data

All microscopical taxa lists have been standardized using the established WFD (EU project WISER) taxa codes, i.e. the VALID code system for diatoms in phytobenthos (LfU) and the REBECCA code for non-diatoms (soft algae) An Access database for all microscopical taxa and the VALID codes assigned has been prepared (EAWLfU, FEM, LFUI). This EAW taxa analysis tool supported the data exploiration.

Results on comparison between traditional microscopy and HTS

Soft algae

There are no soft algae counts by microscopy in the project sampling campaign.
The HTS cyanobacteria results (16S) are very interesting with 27 different taxa found (see in detail in Suppl. Table in appendix).
The most important detected cyanobacteria in phytobenthos of river Wertach were in the family Leptolyngbyaceae. The genera *Pleurocapsa, Chamaesiphon, Calothrix, Schizothrix* and at few stations the potentially toxic producing *Tychonema* were detected by 16S HTS.

Regarding the second gen marker for softalagae, the 18S metaborcoding recorded a very diverse phytobenthos with strongest signals for common diatom and ciliat taxa. Concerning the 18S signals for gree-algae, endophytic chlorophyte taxa such as *Chlorochytrium lemnae,* which live in macropyhtes, dominated over known phytobenthic taxa. At the five Wertach stations between 127 and 299 different genotypes were detected, and these mainly belonging to benthic or pelagic living micro-organisms and not to allochthon vegetation. Macroscopically visible species such as the chrysophyte *Hydrurus foetidus* were present at Wertach stations (but not found before). This species was confirmed in other EAW samples: It was microscopically detected in the Austrian pilot river Steyr. The strong eDNA detection of the Batrachospermales *Sirodotia delicatula* for Wertach station Thalhofen (sample 2) is doubtful, since this species is known only from Japan and Indonesia.

Benthic diatoms
Twenty-three diatoms genera were detected using both methods (42 % of shared genus). Sixteen diatom genera were found through metabarcoding, but were not detected under the microscope, and only four diatom genera were not identified by metabarcoding, but found under the microscope (Table 3.1).

When looking at the taxonomic assignment at the species level, the correspondence between the two methods is lower, with only 14-16 diatom species in common, representing 27% of all species identified by both methods (Fig. 4.2). 20 species were identified only by microscopy, and 25 species only by HTS. The correspondence between the methods is therefore weaker at the species level than at the genus level.

Table 3.1. Comparison of diatoms taxa at genus level for river Wertach detected using the two different methods (microscopical analysis vs sequence analysis HTS) or detected only by one method

common	**only LM**	**only rcbL HTS**
Achnanthidium	Achnanthes	Cyclotella
Amphora	Diadesmis	Discostella
Caloneis	Meridion	Ellerbeckia
Cocconeis	Staurosirella	Fistulifera
Cymatopleura		Frustulia
Cymbella		Gomphonella
Denticula		Hippodonta
Diatoma		Iconella
Diploneis		Karayevia
Eolimna		Luticola
Fragilaria		Parlibellus
Geissleria		Pseudostaurosira
Gomphonema		Sellaphora
Gyrosigma		Staurosira
Mayamaea		Thalassiosira
Melosira		Tryblionella
Navicula		
Nitzschia		
Planothidium		
Reimeria		
Rhoicosphenia		
Surirella		
Ulnaria		

It is noteable, that the rcbL metabarcoding was able to detect a higher number of diatom species (Fig 3.2; N = 102), and in addition, detected diatom signals from biofilm samples, which are usually grow in pelagic samples such as *Cyclotella* and *Thalassiosira* taxa. Latter finding is a hint, that eDNA from the running water was attached to the biofilm of the stones.

When focusing on the finding rates from the perspective of the traditional method applied for implementing the European Water Framework Directive (WFD), 50% of all taxa found by light microscopy were found also by HTS. The other way round, HTS provided a long taxa inventory list including formely undetected species (see appendix Suppl Table 3.4.).

When comparing the proportion of one species in total counted valves to its proportion in total HTS signal (compare Fig. 3.3 to 3.4), this must be done under the precaution, that cells can contain multi-copies of RNA. For example at Wertach station Gogglewehr *Diatoma vulgaris* was found by both methods in all three replicates (bright blue), but with a much higher proportion by HTS. Regarding relative abundance for one diatom species between samples, in accordance both methods did detected *Melosira varians* strongly in replicates 1 and 3, but less in replicate 2 at station Gogglewehr.

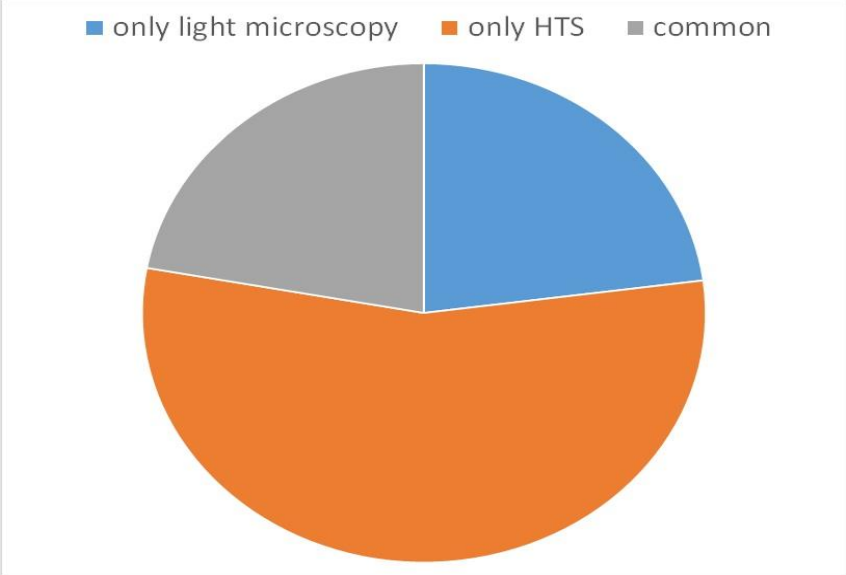


Fig. 3.3. Mean percentage of diatoms species identified by HTS and microscopy (common) (14), only microscopy (only µscopy) (30), or only by HTS (only HTS) (53), for Wertach samples.

In total 80 diatom taxa were found in river Wertach, from which 29 taxa were above 2% of total counts in at least one of the samples (Fig. 3.3). A study by SEM (Goos, 2021) confirmed *Cocconeis placentula var. lineata, Encyonema minutum, Gomphonema parvulum var. parvulum fo. parvulum, Gyrosigma attenuatum*, all identified only by light microscopy in at least one sample.

Extreme smal diatoms such as *Fistulifera saprophila* and *Gomphonema minutum f. minutum* were detected by the HTS method, but were not regognized by light microscopy. The list of **non-corresponding diatom species** identified from biofilm through HTS only (rbcL reference database Diat.barcode v9) from River Wertach is impressive long with 55 additional taxa.

With focus on those samples with strong HTS signal, a special proof by scanning electronic microscopy (SEM) was contracted (Goos 2021) and these checked species are marked in Suppl Tables 2.3 and 2.4. In Wertach *Achnanthidium delmontii, Encyonema ventricosum, Fistulifera saprophila, Gomphonema saprophilum,* and *Gyrosigma sciotense* were confirmed by the SEM study in at least one of the samples. The full report with more than 600 microphotographs is available to the whole project consortium.

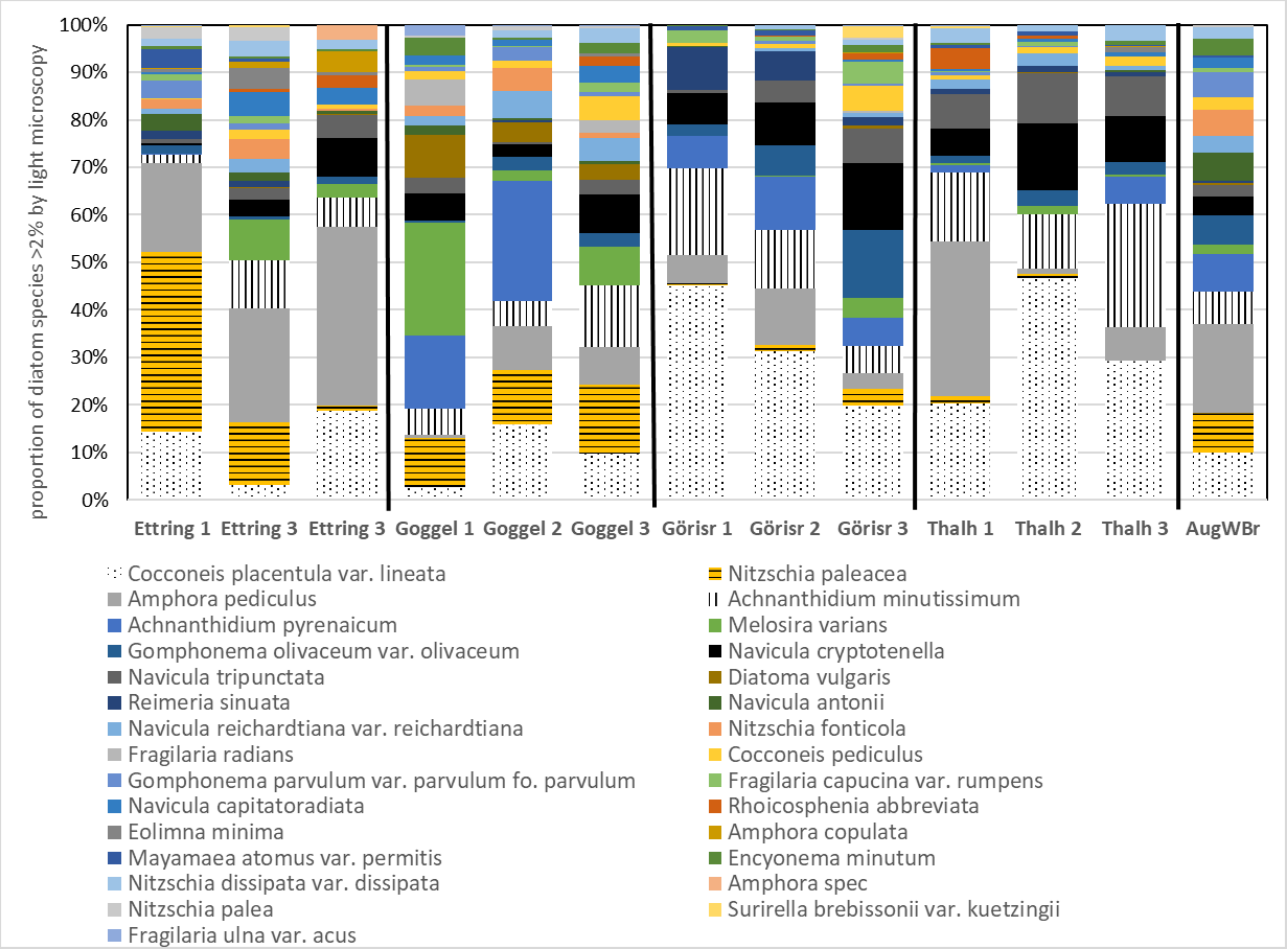


Fig.3.4. Relative abundance of diatoms (> 2% of total counts) at five riverine sampling sites from River Wertach, as revealed from microscopical counting. For geographic location of sites see Fig. 4.1 and notify that 3 replicates at the main stations were sampled done and analyzed seperately.

Especially noteable is the confirmed detection *of Achnanthidium delmontii* in the river Wertach, since this is an invasive species and not found by light microscopy up to now. The scanning electronic documentation of *Achnanthidium delmontii* according characterisation by Pérès et al. (2012) and its discrimination from a very similar species *Achnanthidium pyrenaicum* detected by the light microscopy demonstrates the value of data proof by a multi-proxy approach.

Doubtful HTS signals are for the follwing diatom species: *Diploneis subovalis* is common in brackish waters (Lange-Bertalot et al. 2020). The signal of *Staurosira construens* was very strong in some samples, but not found by SEM or by light microscopy.*Nitzschia dissipata var. media* with low rcbl signal was also not recorded by traditional microscopy.

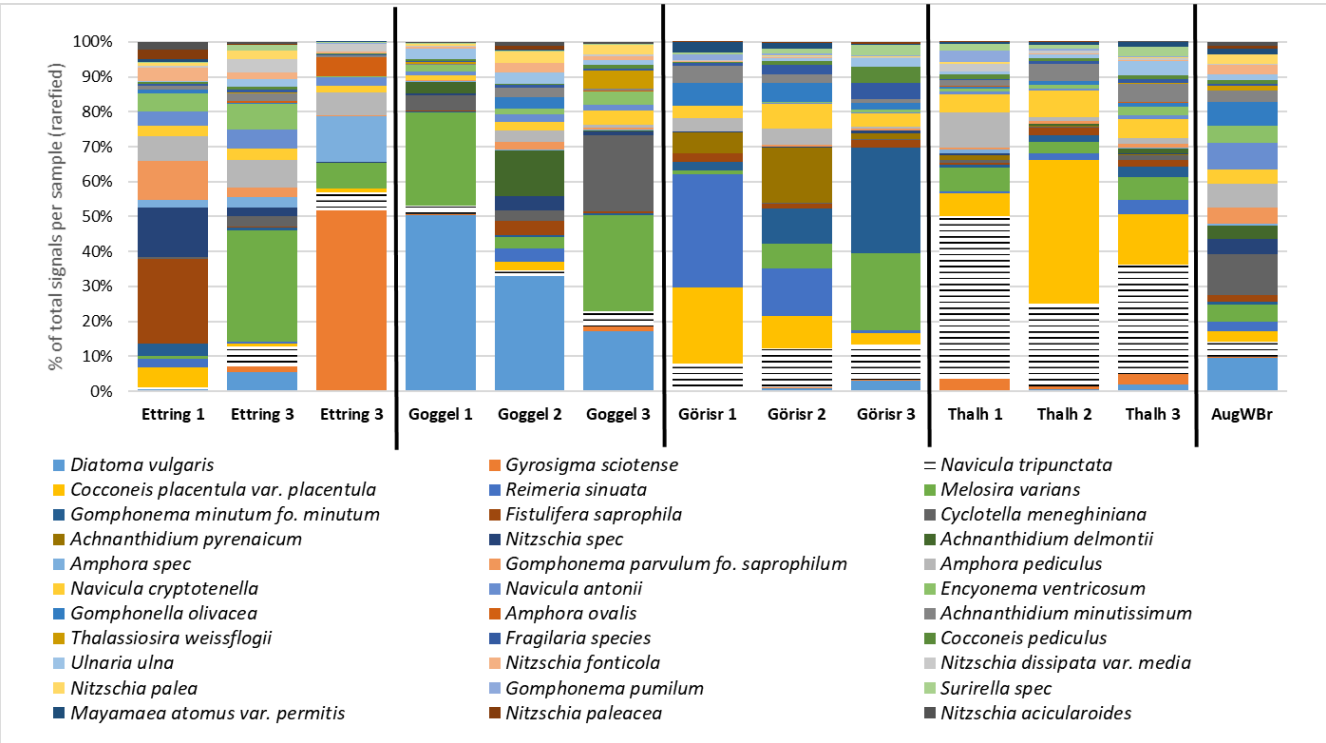


Fig. 3.5. Relative abundance of diatoms reads (> 2% of total reads) at four riverine sampling sites from River Wertach, as revealed from HTS sequencing (for location of sites see Fig. 3.1).

Table 3.2. Detection of invasive species Achnanthidium delmontii by rcbL V9 gen marker in river Wertach with signal values as the sum of genotypes of this species found in one sample.

site_name	station_DB_EAW	HTS signal for taxon	N Variant_ESV_V9
Wertach	WertachBr3_HymC	1908	4
Wertach	P_Thalh.3067_3	624	4
Wertach	P_Thalh.3067_2	283	4
Wertach	P_Thalh.3067_1	48	4
Wertach	Goggel.W3093_3	48	4
Wertach	Goggel.W3093_2	5382	4
Wertach	Goggel.W.3093_1	1513	4
Wertach	Ettring.3074_3	9	2

Dominant species were mainly identified by both methods. However, some species were not identified by metabarcoding. In other cases, a taxon was potentially detectable by HTS, but not found in a sample with high counting value. In particular:

Dominant species, which were not detected by HTS:

Cocconeis lineata Ehrenberg (Syn. *Cocconeis placentula var. lineata*) was very abundant and frequently found in Wertach samples (share of total valves: 3-35%) with light microscopy, but in HTS instead *Cocconeis* spec,

Cocconeis pediculus or *Cocconeis placentula var. placentula* was found with high signal.

Dectable HTS species, but signal missing in some samples:

Diatom taxa dectected at least in one sample by HTS (partly with several genotypes) were marked as "detectable by HTS" in the output tables of the EAW taxa analysis tool.

Achnanthidium pyrenaicum was detected by both methods in several Wertach samples (for example Görisr.3063_2), but in two Goggelewehr samples with high dominance (15-25%) according counting result, there was no detection by HTS. Instead of *A. pyrenaicum*, HTS found the very near related *Achnanthidium delmontii.* In conclusion, within the very similar morphotype there are two hidden taxa, which can easy be confused!

Change of taxa names causes mismatch:

Gomphonema olivaceum var. olivaceum ((Hornemann) Ehrenberg 1838) was missing in the HTS outputs, but there were *Gomphonella olivacea* (Hornemann) Rabenhorst 1853, which is the recently accepted name for the same species. In this case, the EAW taxa anlysis tool is not proper prepared to link the findings of the same species with both methods (update needed for this synonomy).

Calculation of the water quality indices was not done for the eDNA approach but with the microscopical counting result data (tab. 3.3), because too many HTS diatom taxa had no trophic score.

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 with good or moderate startus (see table 3.3).

Tab. 3.3 Biological water quality index for diatoms in the assessment system PHYLIB calculated from counting data for the five river Wertach stations.

Wertach stations (EAW)	diatom river type	assess-ment diatom metric	Index diatom
Goggel.W.3093_1	D 4	moderate	2,6
WertachBr3_HymC	D 4	good	2,24
Görisr.3063_1	D 1.2	moderate	3,16
Ettring.3074_1	D 4	good	2,25
P_Thalh.3067_1	D 1.2	moderate	2,58

Conclusion on results obtained for diatoms

Relevant information derived from sequencing includes the following:

(i) Good match between microscopy and HTS for assignment to genus level, but even on genus level, deep and current taxonomic knowledge is required to link the modern names from HTS taxonomy to names used in traditional counting lists (see *Gomphonema* or *Gomphonella*).

(ii) When looking at the taxonomic assignment at the species level, the correspondence between the two methods is lower, with only 14-16 diatom species in common, representing 27% of all species identified by both methods. Still, most of the dominant species were identified by both methods.

(iii) More work needs to be done to harmonize the traditional and HTS approaches by including missing gen references for taxa commonly found in German rivers, and to better harmonize taxa findings to current nomenclature in modern taxonomy.

3.2 Fish results, River Wertach

Germany (PP7, LfL)

Christian Vogelmann & Michael Schubert

Sampling

The sampling on the River Wertach took place at sampling site 1 (Wertach Görisired) on 14.October 2019 and at the second site (Turkheim uh. Wehr) on 17.October 2019 according to the Eco-AlpsWater protocol D.T1.3.1-4 - Lake and river eDNA fish sample collection from the field for downstream molecular analysis.

VigiDNA®:

Standard sampling: 30 liters of water were taken in the flowing wave with a peristaltic pump system and pumped directly through VigiDNA® 0.45 µm filter cartridges. One filter was used for each sampling stretch (Görisried and Türkheim). After filtration, the cartridges were filled with a preservation buffer and stored in the fridge until DNA extraction according to Eco-Alpswater protocol D.T1.3.1-4 Lake and river eDNA fish sampling. In the meantime, however, we would no longer recommend storing the samples in the refrigerator due to difficulties, especially with regard to DNA extraction. Therefore, it is advised to store the samples at room temperature until extraction.

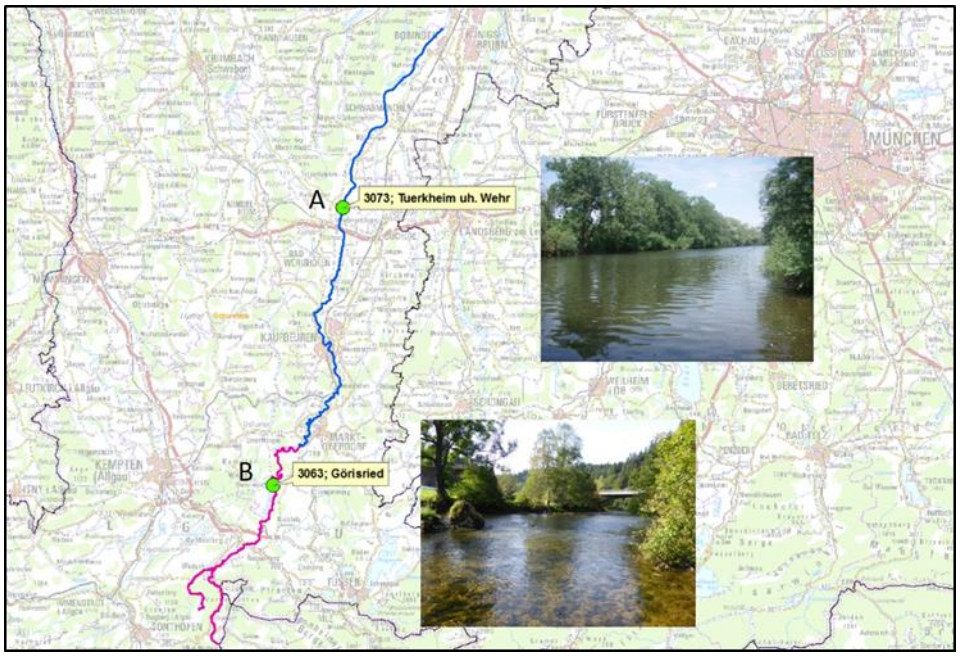


Figure 1: Sampling sites river Wertach. A = Tuerkheim uh. Wehr (2000 m electrofishing stretch by boot). B = Görisried (800 m electrofishing stretch by wading).

DNA extraction and sequencing

For the fish eDNA extraction from VigiDNA® cartridges a combination of the Macherey-Nagel NucleoSpin® and the DNeasy Soil Kit® was used according to the Eco-Alpswater protocol D.T1.3.1-8.2 - Fish DNA extraction from VigiDNA® cartridges. For the fish eDNA extraction from GFC filters, the DNeasy Power Water kit (Qiagen) was used, following the manufacturer's protocol.

The PCR amplification as well as the library preparation was done by AGES (Austrian Agency for Health and Food Safety) according to the the Eco-Alpswater protocol D.T1.3.1-12 - Library preparation 12S. For the sequencing, MiFish-U primers (forward: 5`- GTCGGTAAAACTCGTGCCAGC-3`, reverse: 5`- CATAGTGGGGTAT-CTAATCCCAGTTTG-3`, Miya et al. 2015) were used and for each sample. For each VigiDNA® sample nine replicates were performed, for the GFC filters only one.

Bioinformatic processing

Raw sequencing data were analyzed at the Research Department for Limnology, Mondsee. For the bioinformatics analysis, the qiime2 pipeline (Bolyen et al. 2019) was used. This pipeline was originally designed to work on microbiome data. However, previous test showed, that the taxonomic assignment of the obitools3 pipeline, which was used by most partners in the EAW project, and the taxonomic assignment of the qiime2 pipeline delivered comparable results regarding the taxonomic assignment of fish in eDNA samples. Due to easier handling of the bioinformatics processes and a slightly finer taxonomic resolution, the German and Austrian project partners used the qiime2 approach.

Comparison with traditional fish monitoring

The taxonomic inventories obtained from the bioinformatic analysis were then compared to the dataset obtained from the traditional fish sampling at River Wertach, which was carried out directly after the eDNA approach. The traditional method consisted of electrofishing. The sampling stretch Görisried was fished by wading (total 800 m) and second sampling stretch River Wertach Türkheim was fished by boat (total 2000 m).

Results on comparison between traditional monitoring and HTS

For each VigiDNA® samples, 9 replicates were sequenced. For the analysis, the average number of reads per species (occurring in the 9 replicates) in each sample was determined and then summed up.

Sample site River Wertach Türkheim (Figure 1; B):

A total of 13 fish species were confirmed, 11 thereof by eDNA alone. Two species were detected only with the eDNA approach and not with traditional methods (Perca fluviatilis and Oncorhynchus mykiss).Two Species (Anguilla anguilla and Tinca tinca) were not detected using the eDNA approach but with traditional methods. Barbatula barbatula (1.5%), Abramis abramis (1.4%), Perca fluviatilis (1.4%), Oncorhynchus mykiss (1.4%) and Silurus glanis (0.9%) were found in low proportions.

Table 1: Comparison of fish taxa detected with traditional and eDNA (VigiDNA®) assessment method at sampling site River Wertach Tuerkheim uh. Wehr. The numbers in the molecular method column shows the total number of reads for each species. The traditional methods columns show the number of individuals caught by electrofishing

Common name	Scientific name	eDNA	Traditional methods
Chub	*Squalius cephalus*	12886	335
Schneider	*Alburnoides bipunctatus*	4250	1266
Barbel	*Barbus barbus*	3939	96
Gudgeon	*Gobio gobio*	3584	368
Roach	*Rutilus rutilus*	3146	92
Bleak	*Alburnus alburnus*	1277	297
Stone loach	*Barbatula barbatula*	481	42
Bream	*Abramis abramis*	450	2
Perca	*Perca fluviatilis*	445	0
Rainbow trout	*Oncorhynchus mykiss*	437	0
Wels catfish	*Silurus glanis*	294	43
European eel	*Anguilla anguilla*	0	1
Tench	*Tinca tinca*	0	18

Sample site River Wertach Görisried (Figure 1; B):

A total of 10 fish species were confirmed. With the eDNA approach only 3 fish species could be detected at the sampling site Wertach Görisried. A total of 9 species could be confirmed by electrofishing. One fish species (Rutilus rutilus) could only be detected by the eDNA approach.

Table 2: Comparison of fish taxa detected with traditional and eDNA (VigiDNA®) assessment method at sample site river River Wertach Görisried. The numbers in the molecular method column shows the total number of reads for each species. The traditional methods columns show the number of individuals caught by electrofishing

Common name	Scientific name	eDNA	Traditional methods
Roach	*Rutilus rutilus*	2202	0
Grayling	*Thymallus thymallus*	2176	6
Rainbow trout	*Oncorhynchus mykiss*	2138	24
Brown trout	*Salmo trutta*	0	99
Bullhead	*Cottus gobio*	0	94
Minnow	*Phoxinus phoxinus*	0	92
Schneider	*Alburnoides bipunctatus*	0	8
Chub	*Squalius cephalus*	0	5
Barbel	*Barbus barbus*	0	9
Stone loach	*Barbatula barbatula*	0	2

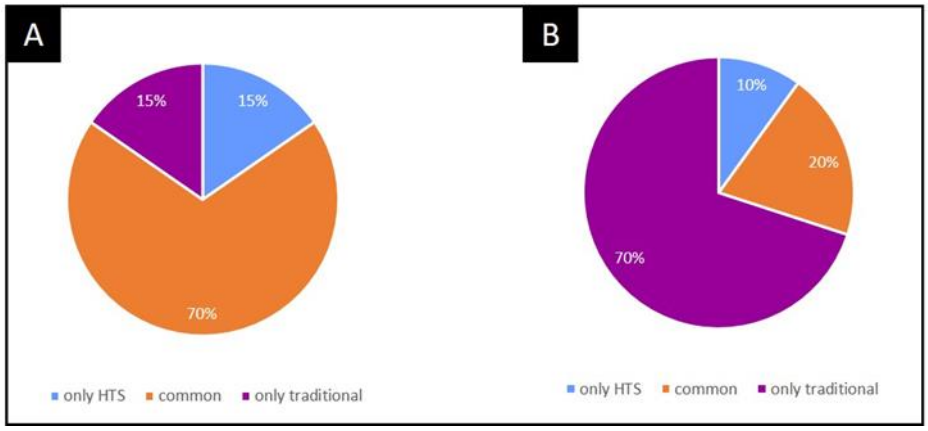


Figure 2 shows the percentage of species detected by VigiDNA® and traditional method (electrofishing). A = sampling site Tuerkheim uh Wehr. B = sampling site Görisried.

Conclusion on results obtained for fish

The eDNA metabarcoding approach shows the potential regarding time and labour effective fish community assessment. For the first sampling site (Tuerkheim) a good overlap between HTS and traditional monitoring methods (68%) was observed. However, at the second sampling site (Görisried) the observed overlap was quite bad, only 20% of species were detected with both approaches. The majority (70%) of species was detected exclusively with traditional methods. This results may be explained due to incorrect storage conditions (fridge) which decreased the DNA retrieval from the filter membrane in the extraction process. In addition, bacterial growth was observed in all VigiDNA® cartridges which led to DNA degradation.

4 River Adige, Italy

4.1 Phytobenthos (benthic diatoms)

Adriano Boscaini[1], Giulia Riccioni[1], Jonas Bylemans[1], Leonardo Cerasino[1], Massimo Pindo[1], Andrea Gandolfi[1], Chiara Zampieri[2] , Federica Giacomazzi[2], Giampaolo Fusato[2], Giorgio Franzini[2], Silvia Menegon[2], Alessia Lea[2], Manuela Cason[2],Nico Salmaso[1].

[1] FEM (PP1)

[2] ARPAV (PP3)

General introduction

The River Adige, a fifth-order river according to Strahler order, is the second longest italian river and the third for catchment area (12,100 km^2). The spring is placed in Val Venosta near the Lake Resia at 1,586 m a.s.l.. The main stream is 409 km long and is spread in the Trentino Alto Adige and Veneto regions, to flow to the Adriatic Sea south of Venice (Distretto Idrografico delle Alpi orientali, 2010).
The River Adige is connected to Lake Garda by the Mori-Torbole tunnel, an artificial underground canal built for flood prevention.
In the northern part of the basin 31 major reservoirs have been built over the last 70 years for hydropower production, with a total capacity of 571 x 10^6 m^3 of water.

Sampling

During the usual agencies BQE monitoring according to the WFD 2000/60. Biofilm (Diatoms) samples were collected in two stations (Arce-Pescantina and Zevio-VR) at 10 October and 5 September 2019 (Fig. 4.1) during low water period according to Deliverable D.T.1.1.2-3 River biofilms sampling.

Diatom samples are taken according to the European Standard: UNI EN 13946:2014 *Water quality-Guidance for the routine sampling and preparation of benthic diatoms from rivers and lakes.*
For the assessment of ecological status, using diatom communities, the Multimeter Intercalibration Index (ICMi) is applied. The ICMi is based on the IPS Pollutant Sensitivity Index and the TI Trophy Index. The Identification of diatoms is at species-level.
For each site, 5 stones were selected along the shoreline. Samples were brushed off from stones from a representative area of 50-100 cm^2 using a clean tray.
From the same stones aliquots, biofilms were preserved in 80% Ethanol as described in protocol (D.T1.3.1-3, River biofilm sampling protocol) in two different tubes, and diatoms were identified both by microscopic analysis and by eDNA analysis.

Rules to define ecological classes and reference conditions

The WFD requires monitoring of diatoms for the assessment of the ecological quality of rivers.
The classification of rivers with diatoms is based on ICMi index based on the recorded species and the attribution of trophic weights of the found species.
The reference method is reported in INTERCALIBRATION COMMON METRIC INDEX - ISS Rapporti ISTISAN 09/2019.

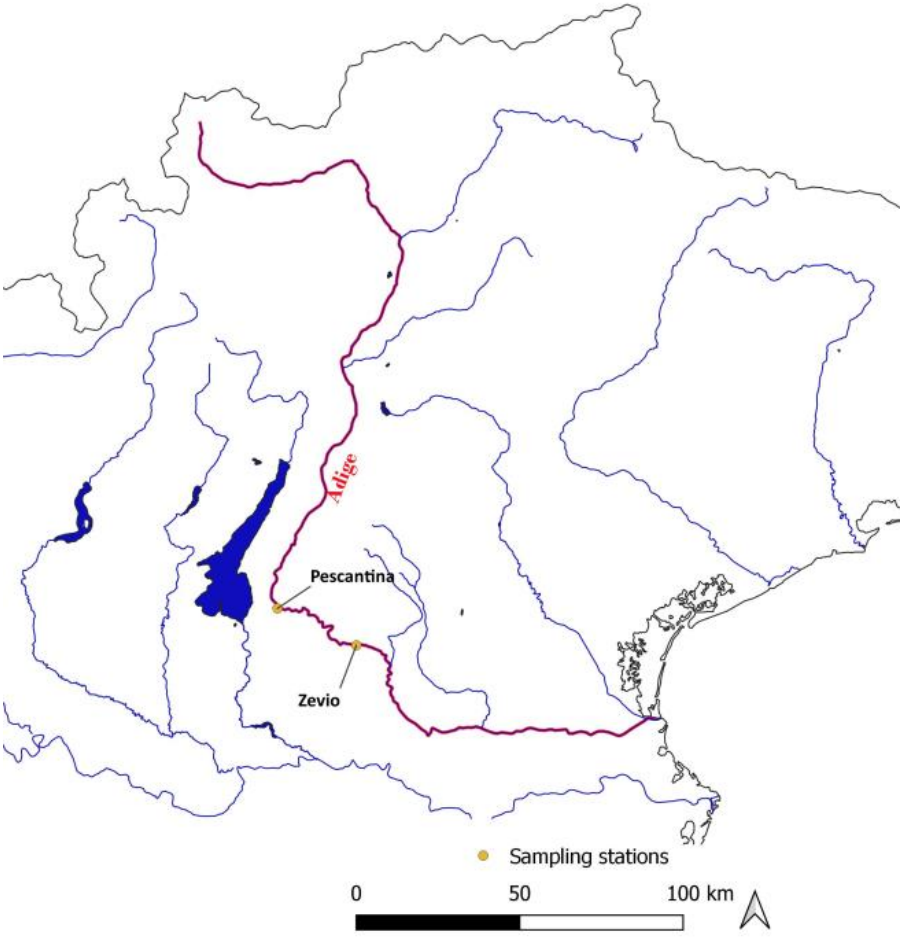


Fig. 4.1 Sampling sites for River Adige (North Italy), Arce-Pescantina e Zevio

Sampling and Results on cyanotoxins concentrations

Aliquots of biofilm were scratched and filtered onto pre-weighed GF/C Filters and the dry-weight was determined from the difference in dried filter (105°C, 24 h) weight before and after filtration. Filters without drying, but stored at -20°C, were then used for cyanotoxins extraction (protocol: Cyanotoxins analyses in lake and biofilm samples).
No cyanotoxins were identified in Arce-Pescantina and Zevio stations.

DNA extraction and sequencing

DNA was extracted using the Machery and Nagel NucleoSpin® Soil kitDNeasy® following the WP1 protocol (DT1.1.2. -7, DNA extraction biofilms).
From sample DNA extracts 16S rDNA (V3-V4 region) has been amplified using primers 341Fmod CCTAYGGGRBGCASCAG and 806Rmod GGACTACNNGGGTATCTAAT under the following conditions: 95°C (5 min), 28 cycles including 95°C (30 sec), 55°C (30 sec), 72°C (30 sec), and final 72°C (5 min). For 18S rDNA (V4 region) the primers V4F-18S_ILL and CCAGCASCYGCGGTAATTCC and V4R-18S_ILL ACTTTCGTTCTTGATYRATGA were applied using the cycling conditions from above. One technical replicate was sequenced (DT1.1.2. -10 Library prep 16S marker gene; DT1.1.2. -11, Library prep 18S marker gene).
PCR amplification and library preparation of purified PCR products for rbcL was performed according to WP1 protocol (DT1.1.2. -9, Library prep RbcL marker gene). Bridge amplification and sequencing by synthesis were performed according to Miseq standard conditions.

Bioinformatic processing

The raw sequence data were processed using the package DADA2, (Protocols DT1.1.3. - 3 Bioinf18S, Bioinformatics treatment 18S marker gene; DT1.1.3. -2 Bioinf16S, Bioinformatics treatment 16S marker gene). Sequences were assigned using the SILVA SSU reference database (bacteria/cyanobacteria) and the PR2 database (protists/microalgae).

For selected ASVs, automated taxa assignment was improved by using reference sequences from relevant taxonomic literature, using (morphologically described) isolates and manual BLAST against ASvs.

For rbcL the raw sequence data were processed using the package DADA2, (D.T1.3.2-1 BioinfRbcL, Bioinformatics treatment rbCL marker gene).

Elaboration of traditional microscopy data

All microscopical taxa lists have been standardized using the established WFD (EU project WISER) taxa codes, i.e. the VALID code system for diatoms in phytobenthos (LfU) and the REBECCA code for non-diatoms (soft algae) An Excel Access database for all microscopical taxa and the VALID codes assigned has been prepared (LfU, FEM, LFUI).

Results on comparison between traditional microscopy and HTS

Soft algae

There are no soft algae counts by microscopy, so there is no data to compare.

Anyway, the HTS cyanobacteria results are very interesting with 22 different taxa found, between these the potentially toxic *Planktothrix*, *Tychonema* and *Phormidium*, (see in detail in Suppl. Table 4.3 in appendix)

Benthic diatoms

Thirteen diatoms genus were detected using both methods (42 % of shared genus). Seventeen diatom genus were found through metabarcoding, but were not detected under the microscope, and only one diatom genus was not identified by metabarcoding, but found under the microscope (Table 4.1).

Table 4.1. Comparison of diatoms taxa at genus level for river Adige detected using the two different methods (microscope analysis vs sequence analysis) or detected only by one method.

common	only microscopy	only HTS
Achnanthidium	Simonsenia	Caloneis
Amphora		Cymatopleura
Cocconeis		Cymbella
Denticula		Diatoma
Encyonema		Didymosphenia
Eolimna		Discostella
Fistulifera		Ellerbeckia
Gomphonema		Fragilaria
Mayamaea		Gomphonella
Navicula		Gyrosigma
Nitzschia		Melosira
Reimeria		Pseudostaurosira
Rhoicosphenia		Sellaphora
		Staurosira
		Surirella
		Tryblionella
		Ulnaria

Among the main genus that are identified by both methods we find *Achnanthidium, Gomphonema, Amphora, Cocconeis, Encyonema, Navicula*. Twenty diatom species were detected by HTS, i.e. sequencing (18S: n=9, rbcL: n=11) and microscopy (Suppl. Table 4.4 in appendix).

Conversely, 13 morphospecies, which were observed at the microscope, were not recorded either via 18S rDNA or rbcL sequencing. Those taxa included morphospecies of the genera A*chnanthidium,* Cocconeis*, Cymbella, Diatoma, Encionema, Gomphonema , Navicula ,Nitzischia, Reimeria* (Suppl. Table 4.5 in appendix). Twenty morphospecies were identified only in sequencing (either through rbcL or 18S rDNA). Those taxa included morphospecies of the genera A*chnanthidium,* Cocconeis*, Gomphonema, Navicula, Nitzschia, Ulnaria* (Suppl. Table 4.6 in appendix).

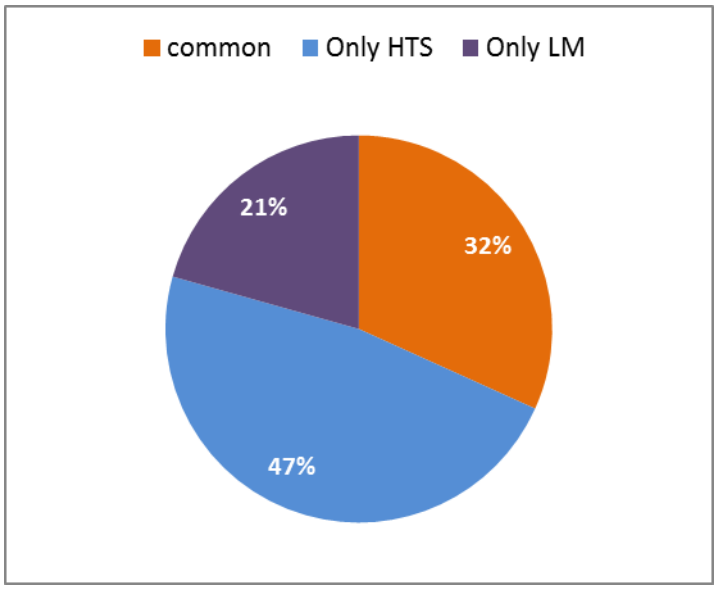


Fig. 4.2. Mean percentage of diatom species identified by HTS and microscopy (common) (20), only microscopy (13), and only by HTS (30), for Adige river samples.

When looking at the taxonomic assignment at the species level, the correspondence between the two methods is lower, with only thirteen diatom species in common, representing 32% of all species identified by both methods (Fig. 4.2). Thirteen species were identified only by microscopy, and thirty species only by HTS. The correspondence between the methods is therefore weaker at the species level than at the genus level.

In samples identified by microscope, *Achnanthidium atomoides*, *Achnanthidium delmontii*, *Achnanthidium microcephalum*, *Achnanthidium* pyrenaicum and Nitzschia fonticola contributed >70% at station 1 (Arce-Pescantina) and *Achnanthidium pyrenaicum* contributed >70% at station 2 (Zevio) (Fig. 4.3).

In samples revealed by HTS *Achnanthidium delmontii*, *Achnanthidium pyrenaicum, Encyonema silesiacum*, and *Nitzschia dissipata* var. *media* contributed >70% at station 1 (Arce-Pescantina) and *Achnanthidium delmontii*, *Achnanthidium pyrenaicum* and *Diatoma vulgaris* contributed 79% at station 2 (Zevio) (Fig. 4.4).

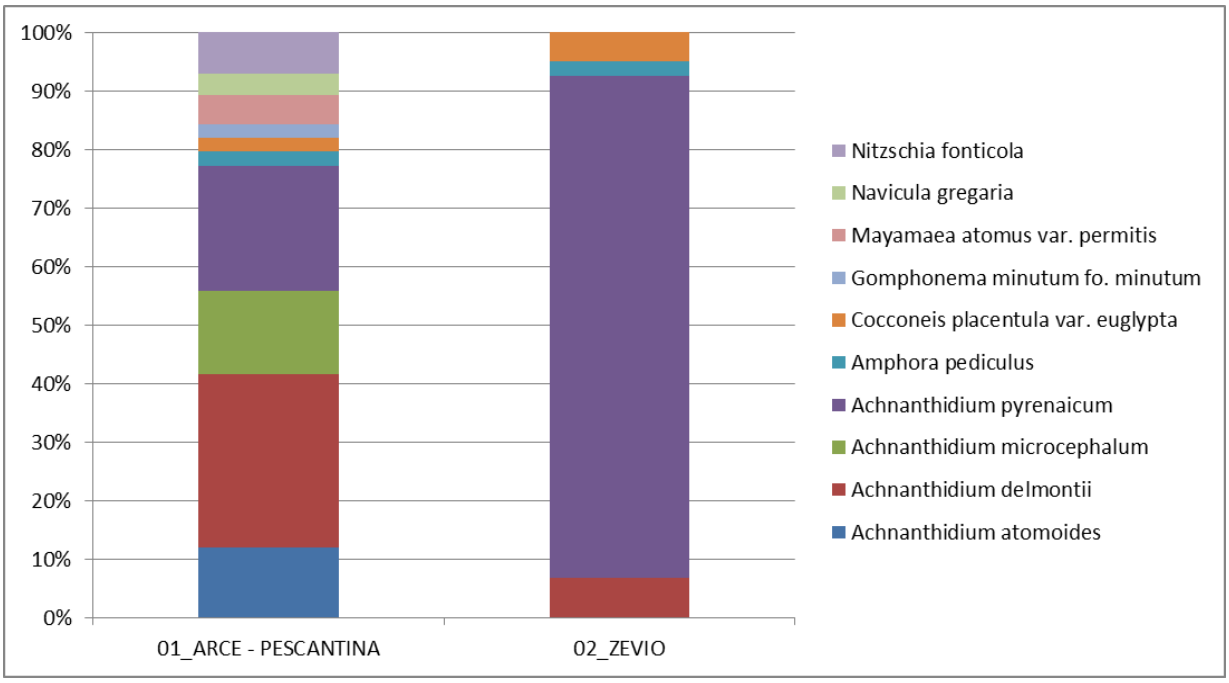


Fig. 4.3. Relative abundance of diatoms (> 2% of total counts) in two riverine sampling sites from River Adige, P1 (Arce-Pescantina); P2 (Zevio) as identified from microscopical counting (for location of sites see Fig. 4.1).

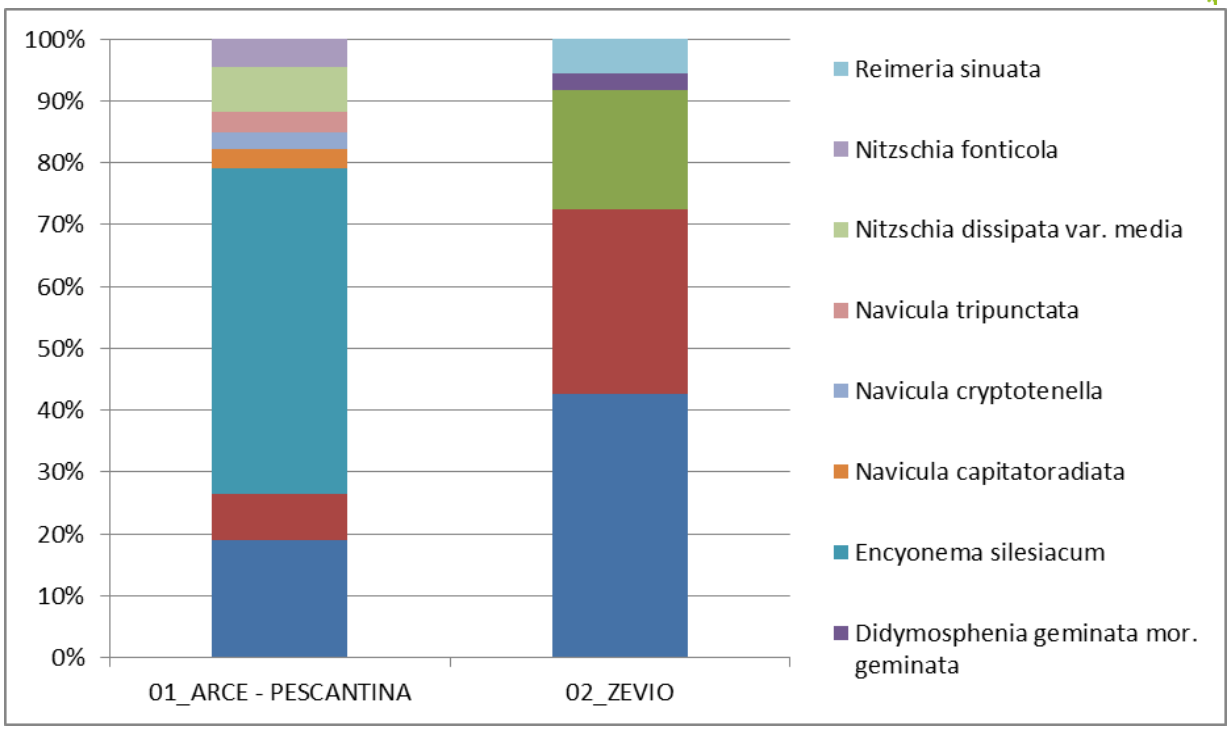


Fig. 4.4. Relative abundance of diatom reads (> 2% of total reads) at two riverine sampling sites from River Adige, as revealed from HTS sequencing (for location of sites see Fig. 4.1).

Conclusion on results obtained for diatoms

Relevant information derived from sequencing includes the following:

(i) Good match between microscopy and HTS for assignment to genus level

(ii) Low match between microscopy and HTS at the species level, but the dominant species were identified by both methods

(iii) Correspondence between microscopy and rbcL or 18S rDNA sequencing is considered useful to confirm microscope based identification of genera

(iv) The diatom taxonomy is constantly evolving with the subdivision of many species into subspecies on the basis of morphological characters. This detail in the classification is often not matched by the HTS.

(v) taxa with low HTS reads abundances have a low probability of being observed under the microscope because the LM reference method is based on the identification of 400 diatoms per slide

(vi) Some species with high confidence of identification are not present in HTS results and others were recorded with more detailed nomenclature in LM

4.2 Fish composition, Adige, Italy

Samplings

Along the Adige river were selected three stations: Vadena in Alto Adige, Mattarello in Trentino and Zevio in Verona province (Fig. 4.5). The samples for fish eDNA analysis were collected on 23 October 2019.

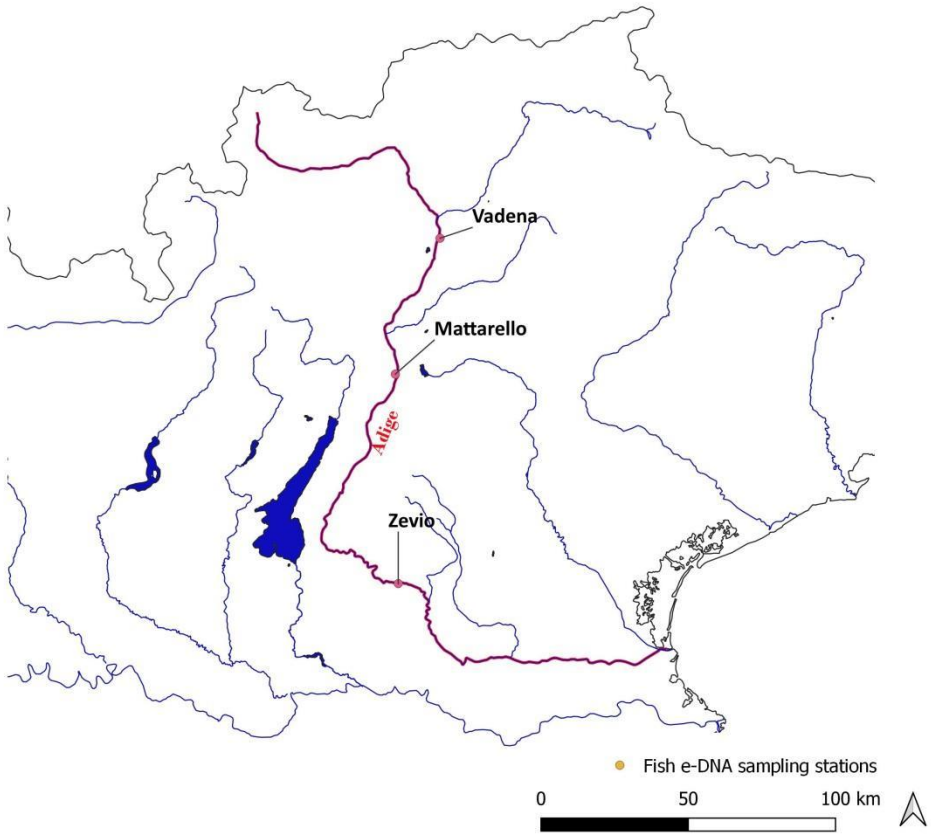


Fig. 4.5. Spatial distribution of fish e-DNA sampling point collected during the 23 October 2019 sampling campaigns.

From the middle of the river, on bridges, in the area of fastest flow, several buckets of water were collected and, after homogenization into a large clean and DNA-free recipient, filtered 30 L on VigiDNA® 0.45 µm capsule. Fish eDNA samples were then preserved in buffer according to the Eco-Alpswater protocol D.T1.3.1-4 Lake and river eDNA Fish sampling.

Traditional monitoring were performed with electrofishing in April 2019 in Mattarello station and in 2003-2004 in Zevio station (Turin et al., 2008).

DNA extraction and sequencing

Fish DNA extraction were performed using the Macherey-Nagel NucleoSpin® Soil kitDNeasy® following the WP1 protocol (D.T1.3.1-8.2, Fish DNA extraction from VigiDNA cartridges).

PCR amplification and library preparation were performed according to WP1 protocol (D.T1.3.1-12, Library preparation 12S) and using the fish specific MiFish-U primers (Miya et al., 2015). Bridge amplification and

sequencing by synthesis were performed according to Miseq standard conditions. Nine PCR replicates were performed for each fish eDNA sample.

Bioinformatic processing

Fish eDNA bioinformatic processing were performed using D.T1.3.2-4 Bioinf_12S protocol from WP1. The protocol uses the OBITOOLS3 software (Boyer et al., 2016,) for the processing of raw high-throughput sequencing reads from the MiSeq platform.

Comparison with fish monitoring

The final output of the eDNA analyses is a tab-delimited table with taxonomic inventories, which is comparable to the species inventories derived from electrofishing catches.

Results on comparison between traditional monitoring and HTS

Unfortunately the HTS sequencing analysis in the three stations identified only one fish species in each station. In the sampling station of Mattarello in the same year was performed one electrofishing campaign in which were collected 6 species, one in common with the HTS *(Squalius squalus*). In Zevio in 2003-2004 was carried out one electrofishing campaign discovering 6 species, one in common with the HTS *(Squalius squalus*; Table 4.2).

Table 4.2. Comparison of fish taxa detected using the two different methods (electofishing vs eDNA sequence analysis). The electrofishing campaign was performed in the site of Mattarello on 3rd April 2019 and in the site of Zevio on 13th February 2003 and 31st May 2004.

Common Name	Scientific Name	site HTS	HTS	Electrofishing 2019 Mattarello	Electrofishing 2003-2004 Zevio
	Alburnus sp.	Vadena	2379		
Chub	*Squalius squalus* (Bonaparte, 1837)	Mattarello	6869	X	X
Rainbow trout	*Oncorhynchus mykiss* (Walbaum, 1792)	Zevio	3342		
Italian barbel	*Barbus plebejus Bonaparte, 1839*			X	x
Brown trout)	*Salmo trutta Linnaeus, 1758*			X	
Marble trout	*Salmo marmoratus Cuvier, 1829*			X	
Common minnow	*Phoxinus phoxinus (Linnaeus, 1758)*			X	X
Bullhead	*Cottus gobio* (Linnaeus, 1758)			X	
Bleak	*Alburnus arborella* (Bonaparte, 1841)				X
European grayling	*Thymallus thymallus* (Linnaeus, 1758)				X
	Padogobius bonelli (Bonaparte, 1846)				X
Eel	*Anguilla anguilla* (Linnaeus, 1758)				X

Conclusion on results obtained for fish

Relevant information derived from sequencing includes the following:

(i) The low number of species detected (one different species for each station) is probably due to the sediment transported by the Adige river that may have hindered DNA extraction.

(ii) In a large river, collect water only in the central point, in the area of faster flow, could exclude species that mainly live near the banks.

5 River Soča, Slovenia

5.1 Phytobenthos (benthic diatoms)

Aleksandra Krivograd Klemenčič, Katarina Novak, Urška Hren (PP5, ARSO)

Phytobenthos has proven to be an indicator of ecological quality status in rivers. In Slovenia, only diatoms (Bacillariophyceae) are used for ecological status assessment. For additional information, we also analyse other phytobenthic algae groups (including Cyanobacteria).

General introduction to the key river

The Soča river (140 km in length) is an Alpine river originating in Trenta Valley in the Julian Alps in northwestern Slovenia, at 990 meters. It flows into the Adriatic Sea. It is best known for its turquoise color, which is the result of dissolved limestone. It has a nival-pluvial regime in its upper course and a pluval-nival regime in its lower course. The Soča river is a torrential river, so its flow fluctuates significantly during the year. In the upper river flow, the difference between the highest and the lowest flow is more than 150-fold; downstream, these differences increase, and in Solkan, the difference between the highest and the lowest flow is as much as 370-fold. In 2019, the annual average water flow in Solkan was 102.3 m^3/s. The torrential character of the river is also visible in the formation of its bed, which is wide and contains an extensive white gravel pit. The river rises very often, moving the gravel down the riverbed and bringing new ones so that the pioneering species of phytobethos cannot overgrow the substrate (past the water meter station Kobarid, Soča river transfers 73.000 m^3 of gravel and 57.000 m^3 of finer material (sand and silt) per year) [1].

The Soča river and its tributaries are used for hydropower generation. Even before the First World War, small hydroelectric power plants (HPP) were constructed on the Soča river tributaries, and later six large HPP were constructed on the Soča river. Tributary Idrijca river brings about 890 kg of mercury into the Soča river every year due to the natural feature of the rocks and 500 years of Idrija's mercury mining.

The entire upper course of the Soča river, from its source to Tolmin, is included in the protected area of Natura 2000, amongst others due to the presence of the Soča trout (*Salmo trutta marmoratus*) and aquatic and waterside habitats.

Sampling according to national legislative

Sampling of phytobentos was performed by the Slovenian Environment Agency (ARSO) according to the standard EN 13946:2014 and national methodology [2].

At each sampling site the distance to the river bank was greater than 1 m and parts of the river with standing water or with extremely low flow were avoided. All available habitats per site (multi-habitat), considering % of substrate type, % velocity, % depth and % shading were sampled. At each sampling site a field datasheet was completed.

The sampled substarata was transferred into a tub together with little river water, where the phytobenthos was scraped with a toothbrush and poured (after mixing) into a labeled bottle with a wide neck. The sample was preserved with alcohol at a final concentration of ~30% for further lab analysis. Under laboratory conditions, the sample was purified with 65 % nitric acid (HNO_3) and heated over a fire until no more organic matter was present. The permanent slides were prepaired using Naphrax and examined according to standard EN 14407:2014 using a light microscope (Leica Leitz DMRB) equipped with a digital camera (Nikon

DS-Fi3). The 500 diatoms' valves were counted in each sample. The abundance of identified taxa was expressed as a percentage. Identification was performed using the identification monographs of Lange-Bertalot et al. (2017)[3] and Krammer and Lange-Bertalot (1986[4], 1988[5], 1991a[6], 1991b[7]).

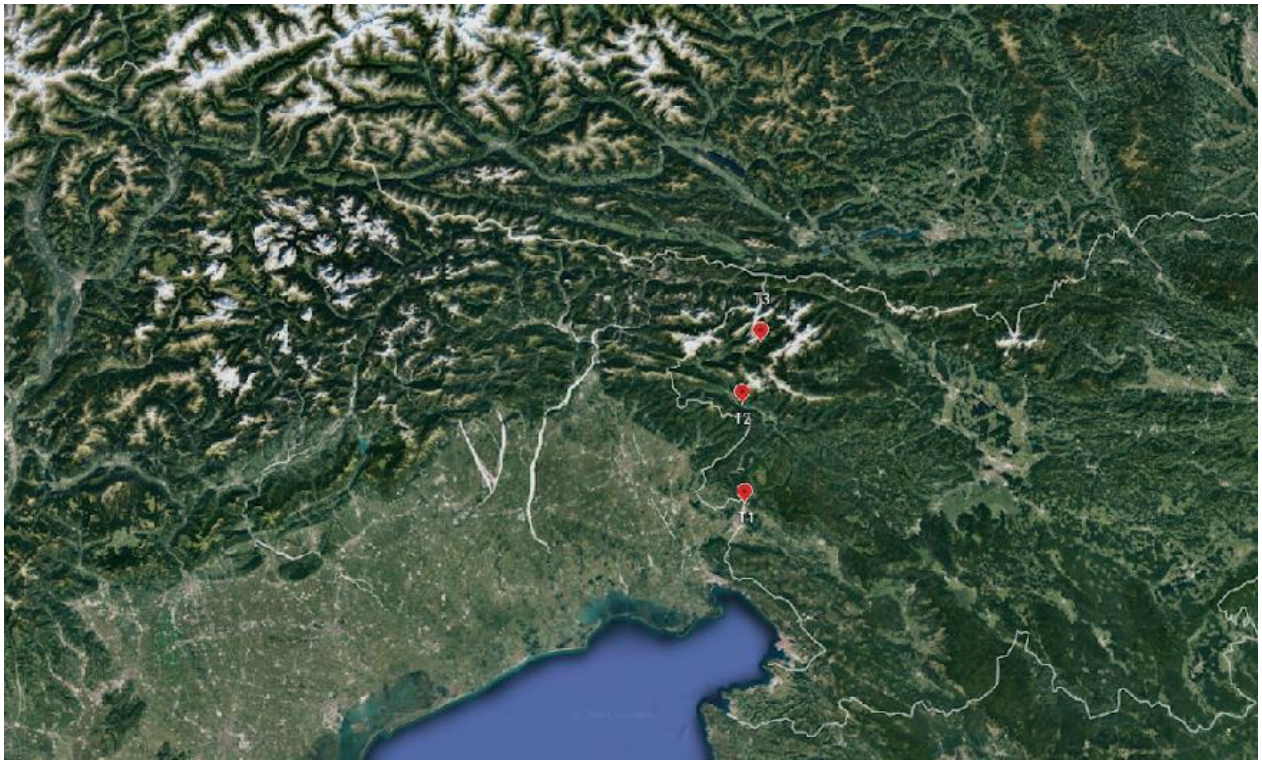


Fig. 5.1. Sampling sites at the Soča river (T1 – Solkanski jez; T2 - Kamno; T3 – Spodnja Trenta)

In parallel with phytobenthos sampling, basic chemical-physical parameters were determined on-site using multimeter (WTW Multi 3630 IDS) and water sample from 0.2 m depth was collected for chemical analysis (pH, conductivity, nitrate nitrogen, sulphates, chloride, calcium, magnesium, sodium, potassium, ammonium itrogen, total nitrogen, total phosphorus, dry weight). The results of chemical analysis showed that total phosphorus (TP) ranged from 9.13 µg P/L (T1) to 19.89 µg P/L (T2). The nitrate-nitrogen (NO_3-N) concentrations ranged from 0.5 mg N/L (T2 and T3) to 0.6 mg N/L (T1), indicating not loaded water.

DNA from the same stones as phytobenthos was sampled was extracted and aliquots were preserved using 80% ethanol as described in protocol (DT1.1.2. -2, Lake biofilms sampling protocol).

Finally, aliquots were scratched directly onto pre-weighed GF/C filters. The dry-weight was determined from the difference in dried filter (105°C, 24 h) weight before and after filtration. Aliquots without drying but stored at -20°C were then used for cyanotoxin extraction (protocol: Cyanotoxins analyses in lake and biofilm samples).

Results on cyanotoxins concentrations

Cyanotoxin anatoxin-a (ATX-a) was detected on Solkanski jez (T1; 0.5 ng/mL) and Kamno (T2; 0.31 ng/mL). Other types of cyanotoxins were not detected at Soča river.

DNA extraction and sequencing

DNA was extracted using the Machery and Nagel NucleoSpin® Soil kitDNeasy® following the WP1 protocol (DT1.1.2. -7, DNA extraction biofilms)

From the sample, DNA extracts 16S rDNA (V3-V4 region) has been amplified using primers 341Fmod CCTAYGGGRBGCASCAG and 806Rmod GGACTACNNGGGTATCTAAT under the following conditions: 95°C (5

min), 28 cycles including 95°C (30 sec), 55°C (30 sec), 72°C (30 sec), and final 72°C (5 min). For 18S rDNA (V4 region) the primers V4F-18S_ILL and CCAGCASCYGCGGTAATTCC and V4R-18S_ILL ACTTTCGTTCTTGATYRATGA were applied using the cycling conditions from above. One technical replicate was sequenced (DT1.1.2. -10 Library prep 16S marker gene; DT1.1.2. -11, Library prep 18S marker gene).

PCR amplification and library preparation of purified PCR products for rbcL was performed according to WP1 protocol (DT1.1.2. -9, Library prep RbcL marker gene). Bridge amplification and sequencing by synthesis were performed according to Miseq standard conditions.

Bioinformatic processing

The raw sequence data were processed using the package Divisive Amplicon Denoising Algorithm 2 (DADA2), (DT1.1.3. - 1 BioinfRbcL, Bioinformatics treatment RbCL marker gene, DT1.1.3. - 3 Bioinf18S, Bioinformatics treatment 18S marker gene, DT1.1.3. -2 Bioinf16S, Bioinformatics treatment 16S marker gene).

Sequences were clustered into ASVs (no dissimilarity threshold) and assigned to the SILVA SSU reference database (or PR2 database?) for taxonomic classification. For rbcL gene assignment to diatom taxa, the curated database R-Syst::diatom (Rimet et al. 2016) was used (INRA).

Comparison with traditional microscopy

All microscopical taxa lists have been standardized using the established WFD (EU project WISER) taxa codes, i.e. the VALID code system for diatoms in phytobenthos (LfU) and the REBECCA code for non-diatoms (soft algae). An Excel Access database for all microscopical taxa and the VALID codes assigned has been prepared (LfU, FEM, LFUI).

In Slovenia only diatoms (Bacillariophyceae) are used for ecological status assessment according to standard EN 14407:2014 and national methodology. For additional information, also other phytobenthic algae groups (including Cyanobacteria) are analysed according to taxa list and relative abundance is estimated using classes from 1 to 5, where 1 is very rare and 5 is dominant.

Results on comparison between traditional microscopy and HTS

Microscopic inspection of phytobenthos samples from the Soča river revealed great difference on **diatom** community between the sampling sites. The diversity of diatom community is increasing downstream, with 28 diatom taxa determined on Spodnja Trenta, 38 diatom taxa on Kamno, and 41 diatom taxa on Solkanski jez (Fig. 5.2). At the upstream sampling point Spodnja Trenta, *Achnanthidium pseudolineare*, *Achnanthidium pyrenaicum*, and *Gomphonema pumilum* were the most dominant taxa. Together, they represent 82 % of all diatoms in the sample. In the sample Kamno, *Cocconeis placentula* var. *placentula* and *Achnanthidium minutissimum* dominated. Together they represent 54% of the identified diatoms. *Encyonopsis microcephala*, *Achnanthidium minutissima* var. *affinis* and *Denticula tenuis* were dominant taxa in the Solkanski jez sample with a total share of 58 %. All three sampling sites are classified as very good ecological saprobic status and very good ecological trophic status.

The results of molecular analyses differ significantly from the results of light microscopy (Fig. 5.3). The abundance of diatom taxa in a sample was just the opposite comparing both methods. In the Spodnja Trenta sample, 42 taxa were identified by molecular analyses (11 of which were also determined by light microscopy), in the Kamno sample, 44 taxa were determined by molecular analyses with 15 common taxa, and in the Solkanski jez sample, only 27 taxa were determined by molecular analyses of which 11 taxa were common with microscopy.

Molecular analyses of Spodnja Trenta sample showed 9 genera (*Adlafia*, *Cyclotella*, *Ellerbeckia*, *Fistulifera*, *Geissleria*, *Iconella Lindavia*, *Mayamaea*, *Staurosira*), which were not registered by light microscopy. Vice versa, species from 5 genera (*Diatoma*, *Aneumastneus*, *Eucocconeis*, *Cymbella*, and *Platessa*) were determined by light microscope, which HTS analyzes did not detect, even though among them are species whose sequences are in the gene bank. The reason for the differences are amongst others also the fact that according to Slovene methodololgy non-benthic cyclic diatoms such as species from genera Cyclotella and Lindavia are not counted.

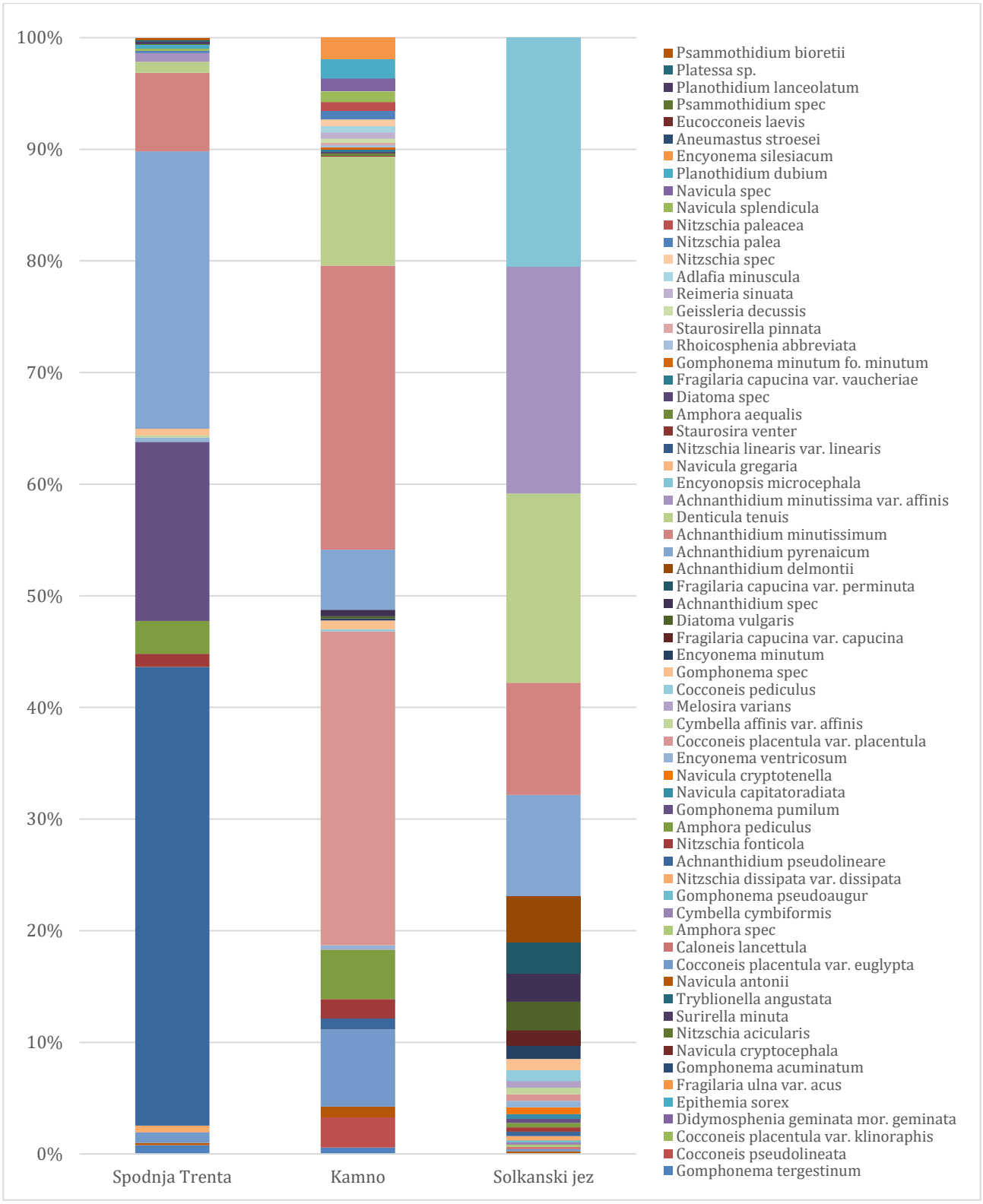


Fig. 5.2. Relative abundance of diatoms collected at three sampling sites at Soča river expressed as percentage from 500 counted valves (for location of sites see Fig. 5.1).

Also, in the Kamno sample, the differences at the level of species and genera are present. Using a light microscope, 7 genera were identified that were not detected by molecular analyses (*Amphora*, *Adlafia*, *Odontidium*, *Geissleria*, *Planothidium*, *Rhoicosphenia*, *Reimeria*). On the other hand, 7 genera detected by HTS analyzes (*Cymbella*, *Caloneis*, *Cyclotella*, *Didymosphenia*, *Discostella*, *Melosira*, *Ulnaria)* were not observed using a light microscope. Again, non-benthic cyclic diatoms such as species from genera *Cyclotella* and *Discotella* are not counted according to Slovene methodology.

In the Solkanski jez sample, despite the large number of taxa identified by the light microscopy, HTS analyzes showed 9 genera that were not detected by the microscopy (*Adlafia*, *Aneumastus*, *Encyonopsis*, *Fistulifera*, *Psammothidium*, *Planothidium*, *Reimeria*, *Staurosira*, and *Ulnaria*). On the other hand, 7 genera detected by microscopy (*Didymosphenia*, *Diatoma*, *Cymbella*, *Epithemia*, *Melosira*, *Surirella*, *Tryblionella*) were not present among the sequences of HTS analyzes. In some cases there is just difference due to different taxa naming. For e.g. *Encyonopsis minuta* (HTS) is the same taxa as *Cymbella microcephala* (microscopy) and *Ulnaria ulna* (HTS) is the same taxa as *Fragilaria ulna* (microscopy), etc. Some other cases are more diffult to explain, such as the presence of big specimens such as *Didymosphenia geminata* detected by microscopy but not by HTS.

Altogether, 67 diatom taxa were determined by microscopy at all three sampling sites and 70 diatom taxa by molecular method of which 29 diatom taxa were common (27%) for both methods. 38 taxa (35%) were identified in the microscopy and were not recorded via 18S rDNA or rbcL sequencing. Vis versa, 41 taxa were identified only by HTS analyses. The first reason for this difference is non-benthic cyclic species' expenses, which are not determined according to the national methodology. We can find some of those species among HTS results (*Cyclotelle distinguenda*, *Cyclotella meneghiniana*, *Lindavia radiosa*, *Discostella woltereckii*, and *Discostella nipponica*). The second reason could be the sampling micro-location (scraping the different part of the biofilm from rocks), but this is unlikely, given that many taxa were detected only by microscopy. The third reason for the difference could be the water flow - the possibility that the eDNA came with the flow of upstream habitats. Finally, we would like to mention that the Soča river is torrential, constantly changing the water level. Along the riverside, the water stagnates and heats up. As a result, there are often species that are not representative of the entire aquatic environment - they show a more eutrophic state, as is it. Therefore, phytobenthos samples are collected at least 1 m from the river banks to avoid those organisms in the samples according to the national methodology. However, the DNA of these organisms is present in the wider water area, which can lead to false results. Such an example could be the presence of *Mayamaea permitis*, *Nitzschia soratensis*, and *Navicula tripunctata*, according to HTS results. These diatoms are common in eutrophic waters, but the Soča river is oligotrophic according to the results of physico-chemical analyses.

Despite the differences in both methods, we can conclude that HTS analyses can serve as good additional information or as means to confirm the microscopy results, especially in diatoms with smaller frustules, where the identification according to the morphology is questionable (e.g., *Achnanthidium minutissium* complex) or in small species that are often overlooked, especially if they are in the girdle view (*Mayamaea permitis*). Additionally, HTS analyses showed the presence of *Achnanthidium delmontii* in the sample Kamno. *A. delmontii* is already considered an invasive alien species in a few European countries[8], so the perception of the species even in small numbers is crucial.

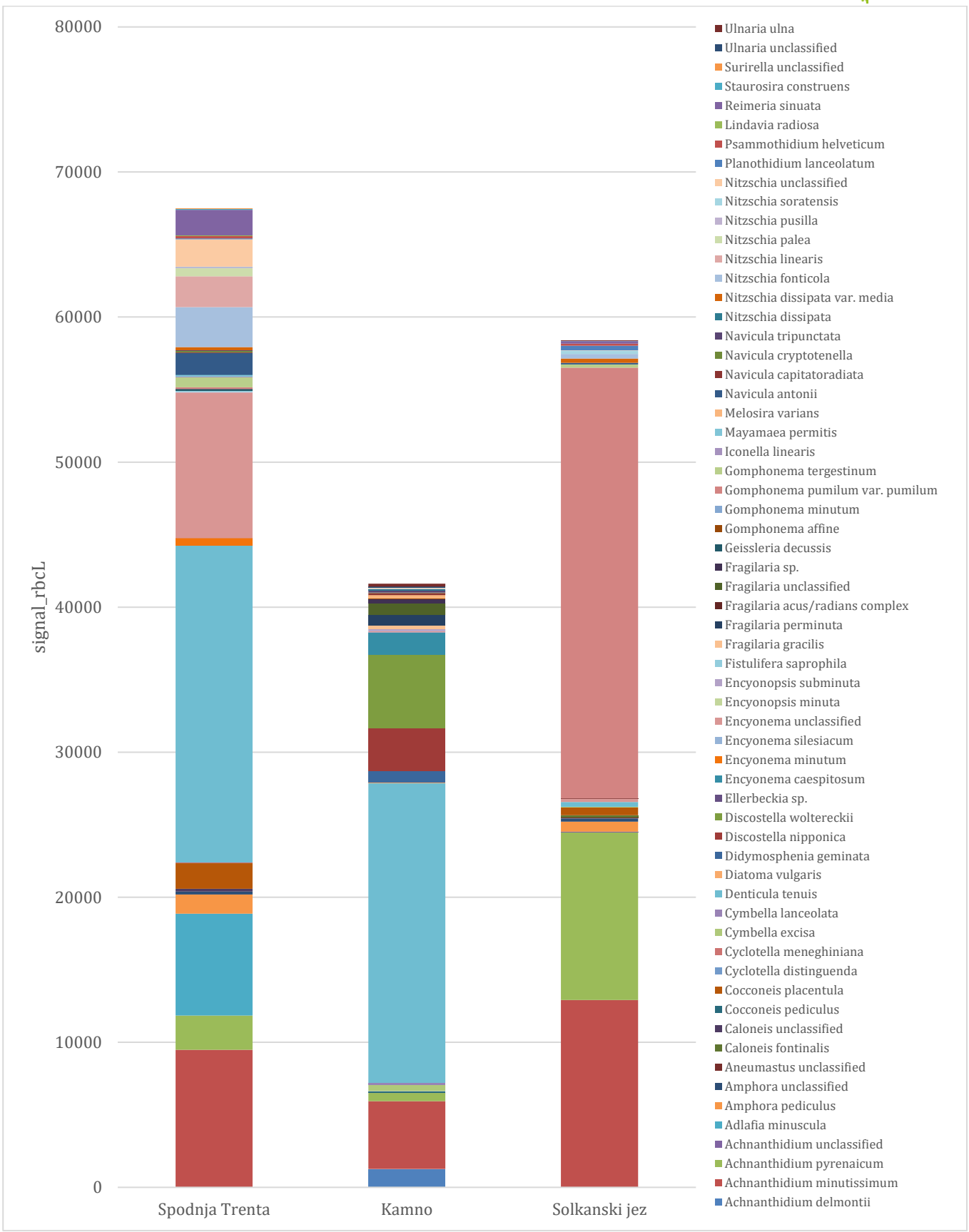


Fig. 5.3. Presence of diatoms at three sampling sites at Soča river according to rcbL signal (HTS analyses) (for location of sites see Fig. 5.1).

Because in Slovenia only diatoms (Bacillariophyceae) are used for ecological status assessment the soft algae are analysed just for additional information and thus taxa and relative abundance determination is not so reliable. As inferred from microscopy within **soft algae**, analyzes showed the presence of representatives of Cyanophyta (50%), Charophyta (33%), and Chlorophyta (17%), all in frequency class 1 in all sampling sites. The highest soft algae taxa diversity was in Solkanski jez sample (*Phormidium* sp., *Cosmarium* sp., *Oedogonium* sp., and *Spirogyra* sp.) Only Cyanophyta representatives were present at the T2 (Kamno) and T3 (Spodnja Trenta) sampling sites, namely 2 on T3 (*Homeotrix varians* and *Phormoidum autumnale*) and 1 on T2 (*Phormidium* sp).

Molecular analyses were more detailed and revealed the presence of multiple soft algae taxa. Most taxa are defined at the genus level. According to the HTS analysis, 33 different taxa of cyanobacteria are present at the Kamno sample site, among them in the largest proportion *Tychonema* sp. (35%). This taxon belongs to the *Phormidiaceae*, which were also observed by light microscopy. Among other soft algae, HTS analyses showed 59 taxa - with the strongest signal were Chlorophyceae (42%) and Ulvophyceae (21%). 10 OU of cyanobacteria and 5 taxa of other solf algae are present at the Solkanski jez sample site. As many as 52% of cyanobacteria were identified only up to the class level, and 38% belong to the Leptolyngbyaceae family. Among other algae that do not belong to the Bacillariophyceae, Chrysophyceae dominates (99.8%). At the Spodnja Trenta sampling site, only 7 taxa of Cyanobacteria were present. The results are similar to those at Solkanski jez, except that 51% of the taxa belongs to Leptolyngbyaceae, and 36% are identified only to the class level. For other algae, 18 taxa were identified. Among them, the class Chrysophyceae dominates (92%).

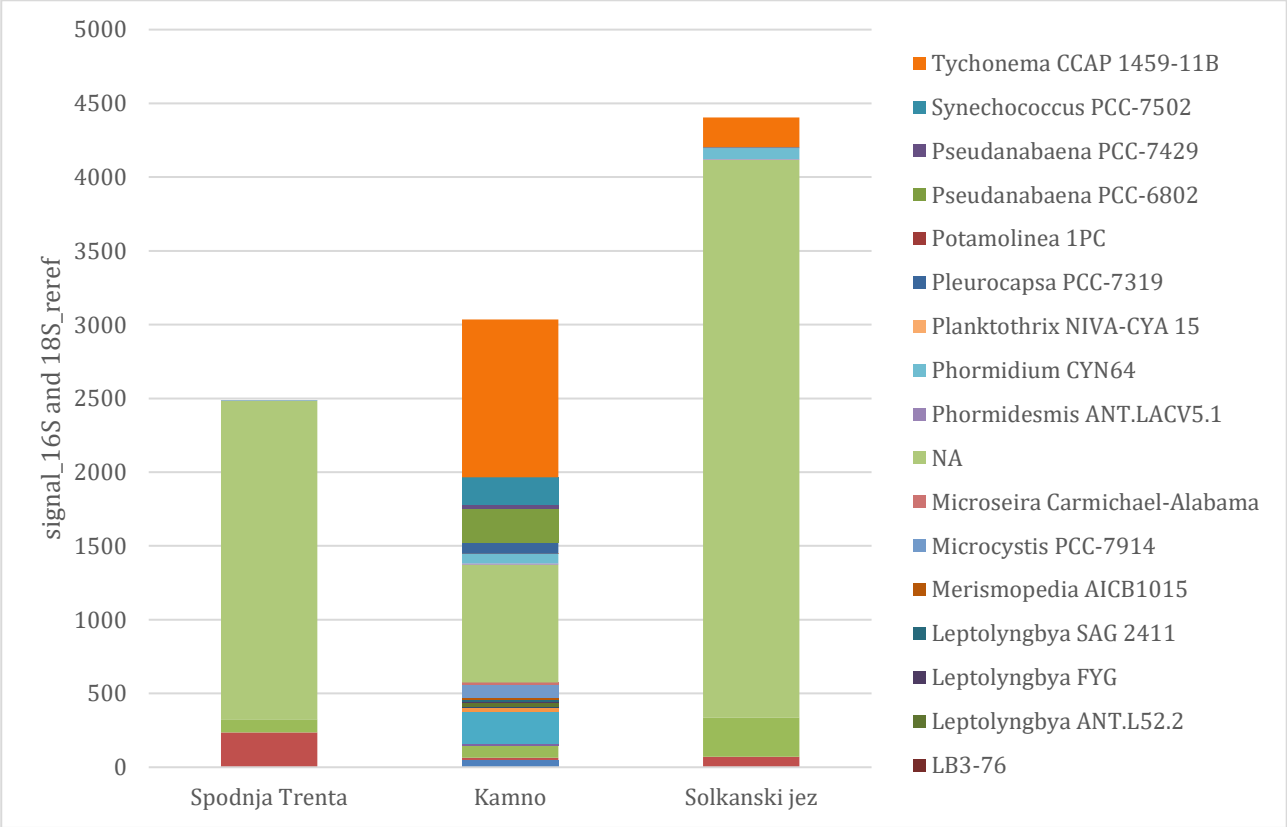


Fig. 5.4. Presence of cyanobacteria and other soft algae (genus level) at sample sites of the Soča river according to HTS analyzes (for location of sites see Fig. 5.1).

Conclusion on results obtained for phytobenthos (cyanobacteria & diatoms)

Relevant information derived from sequencing includes the following:

(i) The 16S rDNA sequencing information can be useful to infer the toxigenic potential of the respective biofilm community, e.g., at site Kamno and Solkanski jez.

(ii) For diatoms, correspondence between microscopy and rbcL or 18S rDNA sequencing is considered useful to confirm microscope-based identification of genera, e.g., for invasive species (*A. delmontii*).

(iii) The results between HTS analyzes, and the traditional method differs significantly, so further studies are needed.

5.2 Fish composition, Soča river

Urška Hren, Katarina Novak, Aleksandra Krivograd Klemenčič, Špela Remec Rekar (PP5, ARSO)

Samplings

FishFish sampling was carried out only in one sampling site – Soča, Kamno (Fig. 5.1.). The samples for fish eDNA analysis were collected on 30 October 2019.

Figure 5.5. Sampling sites for River Soča (T2-Kamno)

At the sampling site Kamno, two sampling replicates were collected. A single sampling replicates consists of collecting and filtering a large volume of water (30 L) from a single sampling site within the mainstream in the area of fastest flow, according to the Eco-Alpswater protocol D.T1.3.1-4 Lake and river eDNA Fish sampling.

After all the water volume had been filtered through the VigiDNA® filter capsule, we added a preservation buffer to preserve the eDNA. The cartridge was placed horizontally, shaken from left to right for 1 minute, and labeled. The filter capsule was placed in the sterile bag and moved into the initial storage box of the filter capsule. Samples were stored in a cooling box.

Traditional fish sampling was carried out by electric fishing in July 2017 in a way that provides a description of the species composition, population assessment (number and biomass of fish), and a description of the size and/or age structure of individual fish species of the investigated section of the watercourse. Comparability of data is ensured to monitor the state of the fish community over time. Each time they shall be sampled at the same place, during the same period of the year, under similar flow conditions, with the same fishing effort, the same fishing gear, and in the same way.

DNA extraction and sequencing

Fish DNA extraction was performed using the Macherey-Nagel NucleoSpin® Soil kitDNeasy® following the WP1 protocol (D.T1.3.1-8.2, Fish DNA extraction from VigiDNA cartridges).

PCR amplification and library preparation were performed according to WP1 protocol (D.T1.3.1-12, Library preparation 12S) and used fish-specific MiFish-U primers (Miya et al., 2015). Bridge amplification and sequencing by synthesis were performed according to Miseq standard conditions. Nine PCR replicates were performed for each fish eDNA sample.

Bioinformatic processing

Fish eDNA bioinformatic processing was performed using D.T1.3.2-4 Bioinf_12S protocol from WP1. The protocol uses the OBITOOLS3 software (Boyer et al., 2016,) for the processing of raw high-throughput sequencing reads from the MiSeq platform.

Comparison with fish monitoring

Fish eDNA bioinformatic processing was performed using D.T1.3.2-4 Bioinf_12S protocol from WP1. The protocol uses the OBITOOLS3 software (Boyer et al., 2016,) for the processing of raw high-throughput sequencing reads from the MiSeq platform.

Results on comparison between traditional monitoring and HTS

By the traditional method (catch), four species were identified: marble trout (Salmo marmorata), European bullhead (Cottus gobio), European grayling (Thymallus thymallus), and rainbow trout (Oncorhynchus mykiss), as shown in Figure 5. The most common species were the European bullhead (79.7% share) and the marble trout (19.4%), representing 99.1% of the fish community in the sampled section of the watercourse. The biomass of these species was estimated at 565.2 g for marble trout (6.4%), 3396.7 g per European bullhead (36.9%), 2.6 g per European grayling, and 5240 g per rainbow trout, which has the largest share of biomass (56.9%).

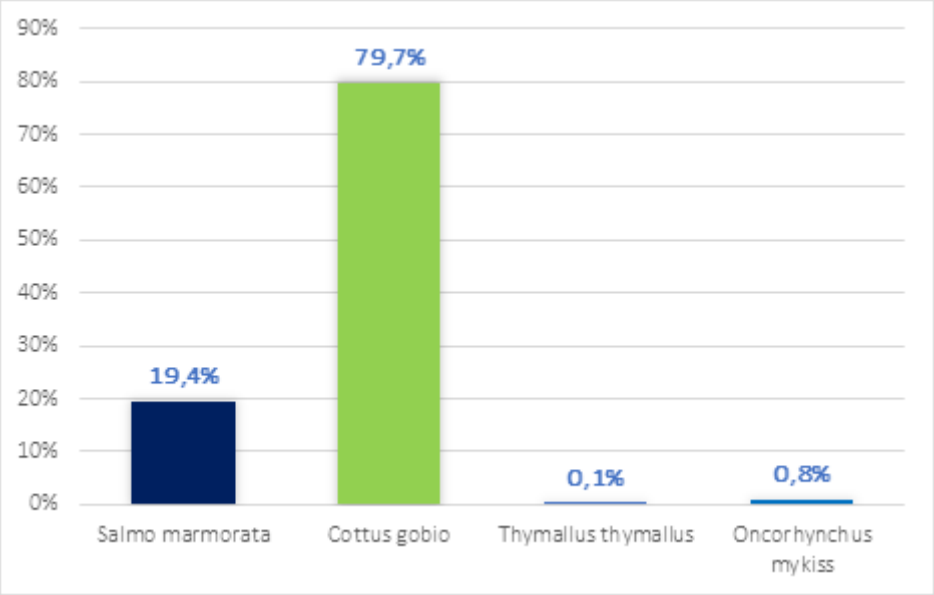


Figure 5.6. Results of traditional method (catch) at sampling site Kamno in the Soča river (2017).

The results of the HTS analysis showed a higher number of species than by the traditional method, as shown in Figure 5.7. The sample is dominated by rainbow trout (Oncorhynchus mykiss), with a share of 45.2%. Species from family Cottidae (36,6 %) could be Cottus gobio, which has the highest share in the traditional method.

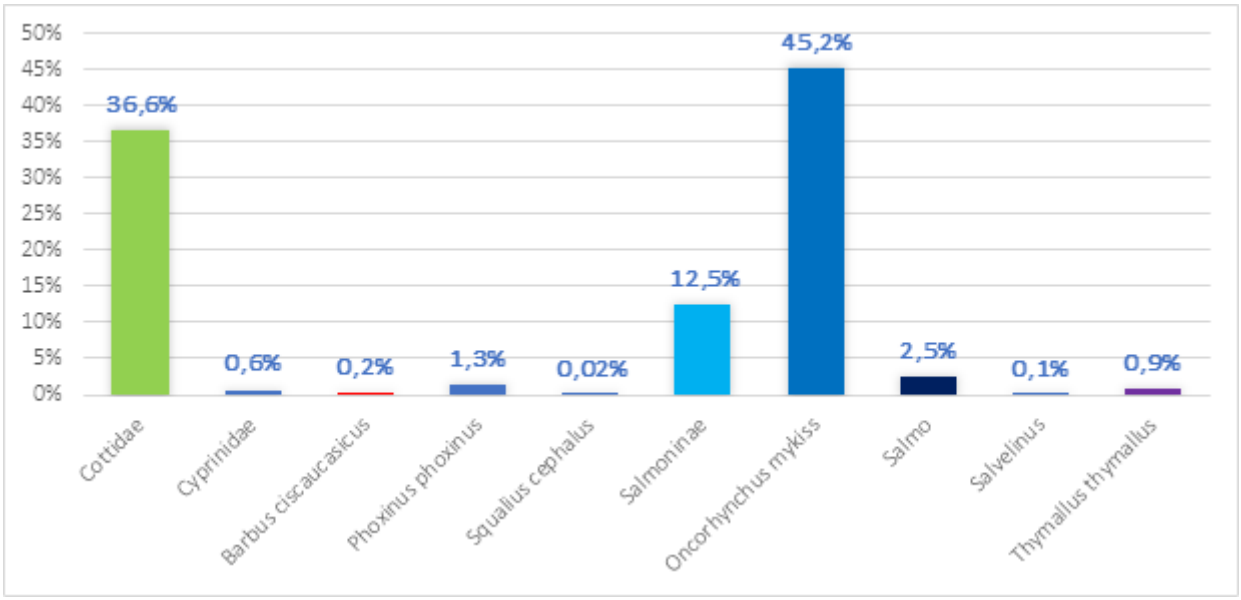


Figure 5.7. Results of the representation of species obtained by the HTS method, for the sample site Kamno in the Soča River, in 2018.

Figure 5.8. shows the results of the proportions of species between the HTS method and the biomass of the catch, where we can see that the proportions of eDNA and biomass between species are similar, at least among those also captured in the catch (Cottidae, Oncorhynchus mykiss, Salmo and Thymallus thymallus). This means that there is a link between biomass and the amount of eDNA. As described above, several species have been identified by the HTS method, but these have very low proportions (those not obtained in the traditional method).

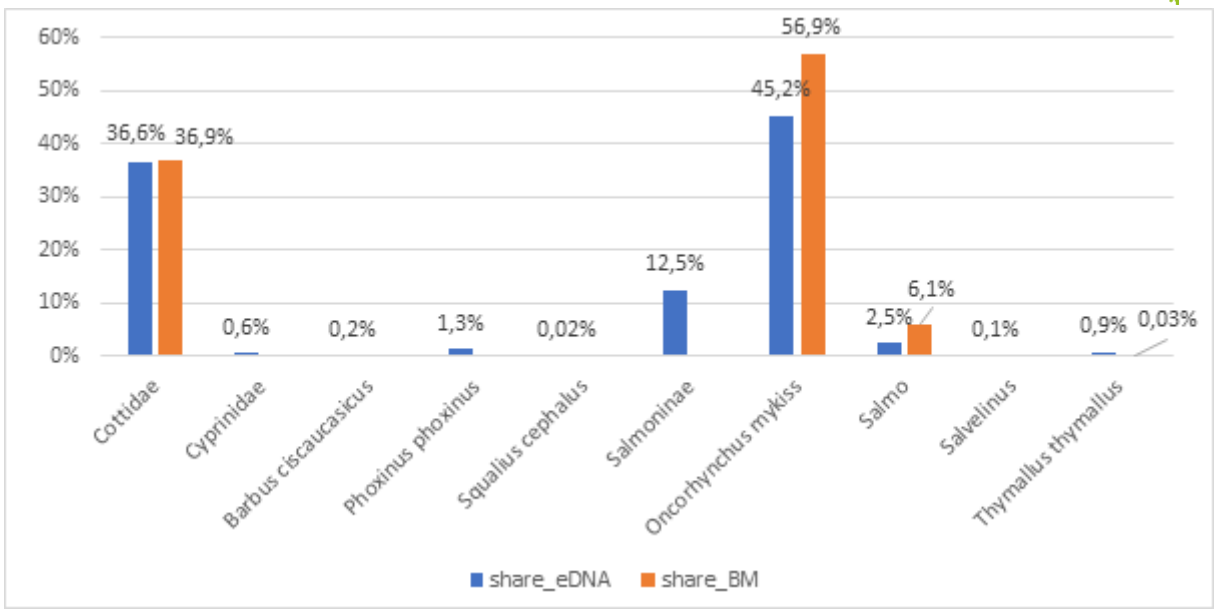


Figure 5.8. Comparison of the HTS method and the traditional method (after a certain biomass) for the sampling site Kamno on the Soča River.

Conclusion on results obtained for fish

Relevant information derived from sequencing includes the following:

i. The HTS method shows more fish species than the traditional method, where there were significantly fewer species / The HTS method gives a better insight into the species representation of fish than the traditional method, as the latter identified significantly fewer species.
ii. A comparison of the HTS method and the biomass of the catch showed that the proportions of fish obtained by both methods were very similar.
iii. The results between HTS analyzes, and the traditional method differ significantly, so further studies are needed.
iv. In most cases, the species most represented in the catch (traditional method) are also represented in the HTS method. Species, which had very low proportions in the HTS analysis, were not obtained (or caught) in the traditional method.
v. Based on the results, it could be said that there is a link between the amount of biomass captured and eDNA.

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6.1 River Steyr, Austria

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7 Appendix (Suppl. Tables)

7.1 River Steyr, Austria

*Suppl Table 1.1. List of **corresponding cyanobacteria** species from **biofilm** identified through microscopy and through HTS (16S rDNA SILVA reference database) from River Steyr (n = 3)*

ID-REBECCA	Taxon_REBECCA	Genus_Rebecca	genus_16S	species_16S
R1518	Synechococcus sp.	Synechococcus	Synechococcus PCC-7502	NA
R1580	Leptolyngbya sp.	Leptolyngbya	Leptolyngbya FYG	NA
R1606	Phormidium sp.	Phormidium	Phormidium CYN64	NA
R1623	Pseudanabaena sp.	Pseudanabaena	Pseudanabaena PCC-6802	NA
R1637	Chamaesiphon sp.	Chamaesiphon	Chamaesiphon PCC-7430	NA
R2006	Pleurocapsa sp.	Pleurocapsa	Pleurocapsa PCC-7319	NA
R2710	Calothrix sp.	Calothrix	Calothrix KVSF5	NA
R2826	Tychonema sp.	Tychonema	Tychonema CCAP 1459-11B	NA
new_16S_cyano_family4	Leptolyngbyaceae		NA	NA
new_16S_cyano_family7	Nostocaceae		NA	NA
new_16S_cyano_family9	Phormidiaceae		NA	NA
new_16S_cyano_family10	Pseudanabaenaceae		NA	NA
biofilm_new4	Schizothrix	Schizothrix	Schizothrix LEGE 07164	NA

*Suppl Table 1.2. List of additional **cyanobacteria** species from **biofilm** identified through HTS (16S rDNA SILVA reference database) from River Steyr (n = 3)*

ID-REBECCA	Taxon_REBECCA	Genus_Rebecca	genus_16S	species_16S
marin2	Aliterella	Aliterella	Aliterella	NA
marin2	Aliterella	Aliterella	Aliterella	NA
biofilm_new6	Phormidesmis	Phormidesmis	Phormidesmis ANT.L52.6	NA
biofilm_new6	Phormidesmis	Phormidesmis	Phormidesmis ANT.LACV5.1	NA

*Suppl Table 1.3. List of **corresponding diatom species** from biofilm identified through microscopy and through HTS (rbcL reference database R-Syst::diatom, 18S rDNA SILVA reference database) from River Steyr (n = 3)*

Locus	V9 species	TAXON_R_Diatom	Validcode
rbcL	Achnanthidium delmontii	Achnanthidium delmontii	newADEL
rbcL	Achnanthidium minutissimum	Achnanthidium minutissimum	ADMI
18S, rbcL	Achnanthidium pyrenaicum	Achnanthidium pyrenaicum	ADPT
18S, rbcL	Amphora pediculus	Amphora pediculus	APED
18S, rbcL	Caloneis fontinalis	Caloneis fontinalis	CFON
rbcL	Caloneis unclassified	Caloneis spec	CALO
rbcL	Cocconeis pediculus	Cocconeis pediculus	CPED
18S, rbcL	Cocconeis placentula	Cocconeis placentula var. placentula	CPLA
rbcL	Cymbella compacta	Cymbella compacta	CCMP
rbcL	Cymbella excisa	Cymbella excisa var. excisa	CAEX
rbcL	Cymbella unclassified	Cymbella spec	CYMB
rbcL	Denticula tenuis	Denticula tenuis	DTEN
rbcL	Didymosphenia geminata	Didymosphenia geminata mor. geminata	DGEM
rbcL	Ellerbeckia sp.	Ellerbeckia spec	ELLE
rbcL	Encyonema caespitosum	Encyonema caespitosum	ECAE
18S, rbcL	Encyonema minutum	Encyonema minutum	ENMI
rbcL	Encyonema prostratum	Encyonema prostratum	EPRO
18S, rbcL	Encyonema silesiacum	Encyonema silesiacum	ESLE
rbcL	Encyonema unclassified	Encyonema spec	ENCY
rbcL	Encyonopsis sp.	Encyonopsis spec	ENCP
rbcL	Fistulifera saprophila	Fistulifera saprophila	FSAP
18S, rbcL	Hannaea arcus	Fragilaria arcus var. arcus	FARC
rbcL	Fragilaria acus/radians complex	Fragilaria radians	FRAD
18S, rbcL	Fragilaria unclassified	Fragilaria spec	FRAG
rbcL	Gomphonella olivacea	Gomphonella olivacea	GLOV
rbcL	Gomphonella olivaceoides	Gomphonema olivaceum var. olivaceoides	GOOL
rbcL	Gomphonema pumilum var. pumilum	Gomphonema pumilum	GPUM
18S, rbcL	Gyrosigma sciotense	Gyrosigma sciotense	GSCI
rbcL	Iconella linearis	Iconella sp.	ICON
rbcL	Mayamaea permitis	Mayamaea atomus var. permitis	MAPE
rbcL	Melosira varians	Melosira varians	MVAR
rbcL	Navicula antonii	Navicula antonii	NANT
18S, rbcL	Navicula cryptotenella	Navicula cryptotenella	NCTE
rbcL	Navicula gregaria	Navicula gregaria	NGRE
rbcL	Navicula unclassified	Navicula spec	NAVI
18S, rbcL	Navicula tripunctata	Navicula tripunctata	NTPT
rbcL	Nitzschia dissipata var. media	Nitzschia dissipata var. media	NDME
18S, rbcL	Nitzschia fonticola	Nitzschia fonticola	NFON
rbcL	Nitzschia soratensis	Nitzschia soratensis	newNSOR
rbcL	Nitzschia unclassified	Nitzschia spec	NITZ
rbcL	Planothidium lanceolatum	Planothidium lanceolatum	PTLA
rbcL	Psammothidium helveticum	Psammothidium helveticum	PHEL
rbcL	Lindavia radiosa	Puncticulata radiosa	PRAD
18S, rbcL	Reimeria sinuata	Reimeria sinuata	RSIN
rbcL	Rhoicosphenia abbreviata	Rhoicosphenia abbreviata	RABB
rbcL	Staurosira construens	Staurosira construens	SCON
rbcL	Ulnaria ulna	Ulnaria ulna	UULN

*Suppl Table 1.4. List of **non-corresponding diatom species** from microscopy to HTS (18S rDNA SILVA reference database) from River Steyr (n = 3).*

Taxon_validcode	Validcode
Achnanthidium lineare	ACLI
Achnanthidium minutissima var. affinis	ADMF
Achnanthidium zhakovschikovii	newAZHA
Amphora copulata	ACOP
Cocconeis pediculus	CPED
Amphora inariensis	AINA
Cocconeis placentula var. lineata	CPLI
Cocconeis placentula var. euglypta	CPLE
Cocconeis pseudolineata	COPL
Cymbella excisa var. excisa	CAEX
Cymbella perparva	CPPV
Cymbella subhelvetica	CSBH
Diatoma ehrenbergii	DEHR
Encyonema ventricosum	ENVE
Encyonopsis minuta	ECPM
Fragilaria candidagilae	newFCAG
Fragilaria capucina var. rumpens	FCRP
Fragilaria capucina var. vaucheriae	FCVA
Fragilaria pectinalis	newFPEC
Geissleria acceptata	GACC
Gomphonema angustum	GANT
Gomphonema angustivalva	GAGV
Gomphonema pumilum var. elegans	GPEL
Gomphonema tergestinum	GTER
Navicula exilis	NEXI
Navicula spec	NAVI
Ulnaria ulna	UULN
Reimeria uniseriata	RUNI

7.2 River Drome, France

*Suppl Table 2.1. List of **corresponding diatom species** identified from biofilm through microscopy and through HTS (rbcL reference database Diat.barcode v7) from River Drôme (n = 4)*

Common	Validcode	ID
Achnanthidium delmontii	newADEL	0
Achnanthidium eutrophilum	ADEU	2419
Achnanthidium minutissimum	ADMI	2438
Achnanthidium pyrenaicum	ADPT	2441
Amphora pediculus	APED	2890
Diatoma moniliformis	DMON	96
Encyonema silesiacum	ESLE	4288
Fistulifera saprophila	FSAP	4585
Fragilaria gracilis	FGRA	201
Fragilaria spec	FRAG	266

Gomphonema tergestinum	GTER	5097
Gomphonema spec	GOMP	5068
Mayamaea permitis	#N/A	#N/A
Nitzschia fonticola	NFON	8679
Nitzschia palea	NPAL	8893
Ulnaria ulna	UULN	672

*Suppl Table 2.2. List of **non corresponding diatom species** identified from biofilm through microscopy only (rbcL reference database Diat.barcode v7) from River Drôme (n = 4)*

Common	Validcode	ID
Achnanthidium lineare	ACLI	2432
Cymbella excisa var. excisa	CETG	3531
Cymbella excisiformis var. excisiformis	CEXF	3536
Encyonema minutum	ENMI	4218
Fragilaria capucina var. vaucheriae	FCVA	175
Fragilaria pectinalis	newFPEC	0
Gomphonema angustius	#N/A	#N/A
Gomphonema angustivalva	GAGV	4793
Gomphonema elegans	#N/A	#N/A
Gomphonema lateripunctatum	GLAT	4920
Gomphonema micropus var. micropus	GMIC	4949
Gomphonema minutum	#N/A	#N/A
Gomphonema olivaceum	#N/A	#N/A
Gomphonema parvulum var. parvulum f. parvulum	#N/A	#N/A
Gomphonema pumilum var. rigidum	GPRI	5032
Gomphonema tenoccultum	#N/A	#N/A
Nitzschia archibaldii	NIAR	8536
Nitzschia paleacea	NPAE	8896
Psammothidium lauenburgianum	PLAU	2708
Ulnaria danica	#N/A	#N/A

*Suppl Table 2.3. List of **non corresponding diatom species** identified from biofilm through HTS only (rbcL reference database Diat.barcode v7) from River Drôme (n = 4)*

Common	Validcode	ID
Caloneis spec	CALO	3263
Cocconeis placentula	#N/A	#N/A
Cymbella spec	CYMB	3677
Cymbella cymbiformis	CCYM	3514
Diatoma vulgaris	DVUL	111
Encyonema ventricosum	ENVE	4342
Gomphonema micropus	#N/A	#N/A
Gomphonema pumilum var. pumilum	#N/A	#N/A
Gomphonema rosenstockianum	GROS	5049
Gomphonema saprophilum	#N/A	#N/A
Melosira varians	MVAR	1670
Navicula cryptotenella	NCTE	5774
Navicula tripunctata	NTPT	6764
Nitzschia spec	NITZ	8993

Nitzschia acicularoides	#N/A	#N/A
Nitzschia capitellata	NCPL	8582
Nitzschia dissipata var. dissipata	NDIS	8630
Nitzschia draveillensis	NDRA	8643
Nitzschia gracilis	NIGR	8710
Nitzschia pusilla	NIPU	8935
Reimeria sinuata	RSIN	7965
Surirella minuta	SUMI	9349
Ulnaria acus	#N/A	#N/A

7.3 River Wertach, Germany

Suppl. Table 3.1. List of cyanobacteria species and genera from biofilm identified through HTS (16S rDNA SILVA reference database) from stations in River Wertach (n = 5)

ID-REBECCA	Taxon_REBECCA	genus_16S	species_16S	Max signal 16S
aerophytic2	Chroococcidiopsis	Chroococcidiopsis PCC-6712	NA	38
biofilm_new4	Schizothrix	Schizothrix LEGE 07164	NA	147
biofilm_new6	Phormidesmis	Phormidesmis ANT.LACV5.1	NA	38
marin2	Aliterella	Aliterella	NA	3
new_16S_cyano_family10	Pseudanabaenaceae	NA	NA	10
new_16S_cyano_family12	Xenococcaceae	NA	NA	33
new_16S_cyano_family4	Leptolyngbyaceae	NA	NA	1766
new_16S_cyano_family7	Nostocaceae	NA	NA	1
Picoplank1	Geminocystis	Geminocystis PCC-6308	NA	6
R1427	Aphanothece clathrata	Cyanobium PCC-6307	NA	1
R1496	Microcystis sp.	Microcystis PCC-7914	NA	1
R1518	Synechococcus sp.	Synechococcus PCC-7502	NA	1
R1580	Leptolyngbya sp.	Leptolyngbya PCC-6306	NA	14
R1580	Leptolyngbya sp.	Leptolyngbya SAG 2411	NA	5
R1606	Phormidium sp.	Phormidium CYN64	NA	1
R1623	Pseudanabaena sp.	Pseudanabaena PCC-6802	NA	6
R1623	Pseudanabaena sp.	Pseudanabaena PCC-7429	frigida	32
R1637	Chamaesiphon sp.	Chamaesiphon PCC-6605	minutus	11
R1637	Chamaesiphon sp.	Chamaesiphon PCC-6605	NA	3
R1637	Chamaesiphon sp.	Chamaesiphon PCC-7430	NA	327
R1637	Chamaesiphon sp.	Chamaesiphon PCC-7430	subglobosus	84
R2006	Pleurocapsa sp.	Pleurocapsa PCC-7319	NA	925
R2302	Cyanobium sp.	Cyanobium PCC-6307	gracile	6
R2302	Cyanobium sp.	Cyanobium PCC-6307	NA	16
R2710	Calothrix sp.	Calothrix KVSF5	NA	341
R2710	Calothrix sp.	Calothrix PCC-6303	NA	6
R2826	Tychonema sp.	Tychonema CCAP 1459-11B	NA	112

*Suppl Table 2.2. List of **corresponding diatom species** (N=39) identified from biofilm through microscopy and through HTS (rbcL reference database Diat.barcode v9) from River Wertach (n = 5). Proof by scanning electronic microscopy (SEM; Goos 2021) is marked with yes in field "SEM detection", if taxon is confirmed.*

LM diatoms BFM	LM Validcode	V9 species name	SEM detection
Achnanthidium minutissimum	ADMI	Achnanthidium minutissimum	
Achnanthidium pyrenaicum	ADPT	Achnanthidium pyrenaicum	
Amphora ovalis	AOVA	Amphora ovalis	
Amphora pediculus	APED	Amphora pediculus	
Amphora spec	AMPH	Amphora unclassified	
Cocconeis pediculus	CPED	Cocconeis pediculus	
Denticula tenuis	DTEN	Denticula tenuis	
Diatoma moniliformis	DMON	Diatoma moniliformis	
Diatoma vulgaris	DVUL	Diatoma vulgaris	
Encyonema caespitosum	ECAE	Encyonema caespitosum	
Encyonema prostratum	EPRO	Encyonema prostratum	
Encyonema silesiacum	ESLE	Encyonema silesiacum	
Fragilaria radians	FRAD	Fragilaria acus/radians complex	
Geissleria decussis	GDEC	Geissleria decussis	
Gomphonema minutum fo. minutum	GMIN	Gomphonema minutum	YES
Gomphonema tergestinum	GTER	Gomphonema tergestinum	
Gomphonema spec	GOMP	Gomphonema unclassified	
Gyrosigma acuminatum	GYAC	Gyrosigma acuminatum	
Amphora montana	AMMO	Halamphora montana	
Mayamaea atomus var. permitis	MAPE	Mayamaea permitis	YES
Melosira varians	MVAR	Melosira varians	
Navicula antonii	NANT	Navicula antonii	
Navicula capitatoradiata	NCPR	Navicula capitatoradiata	
Navicula cryptocephala	NCRY	Navicula cryptocephala	
Navicula cryptotenella	NCTE	Navicula cryptotenella	
Navicula gregaria	NGRE	Navicula gregaria	
Navicula lanceolata	NLAN	Navicula lanceolata	
Navicula tripunctata	NTPT	Navicula tripunctata	
Nitzschia amphibia fo. amphibia	NAMP	Nitzschia amphibia	
Nitzschia dissipata var. dissipata	NDIS	Nitzschia dissipata	YES
Nitzschia dissipata var. media	NDME	Nitzschia dissipata var. media	YES
Nitzschia fonticola	NFON	Nitzschia fonticola	
Nitzschia linearis var. linearis	NLIN	Nitzschia linearis	
Nitzschia palea	NPAL	Nitzschia palea	YES
Nitzschia paleacea	NPAE	Nitzschia paleacea	YES
Planothidium lanceolatum	PTLA	Planothidium lanceolatum	
Reimeria sinuata	RSIN	Reimeria sinuata	
Rhoicosphenia abbreviata	RABB	Rhoicosphenia abbreviata	
Ulnaria ulna	UULN	Ulnaria ulna	

*Suppl Table 3.3. List of **non-corresponding diatom species** from light microscopy (LM= to HTS (18S rDNA SILVA reference database) from River Wertach (n = 5). Proof by scanning electronic microscopy (SEM; Goos 2021) is marked with yes in field "SEM detection", if taxon is confirmed.*

LM diatoms BFM found only by counting	LM Validcode	SEM detection
Achnanthes ploenensis var. woldstedtii	APWO	
Achnanthidium atomoides	ADAM	
Achnanthidium rosenstockii	newADRK	
Amphora copulata	ACOP	unclear
Caloneis lancettula	CLCT	
Cocconeis neothumensis	CNTH	
Cocconeis placentula var. euglypta	CPLE	
Cocconeis placentula var. lineata	CPLI	YES
Cymatopleura solea var. apiculata	CSAP	
Cymbella parva	CPAR	
Diadesmis contenta	DCOT	
Diploneis separanda	DSEP	
Encyonema minutum	ENMI	YES
Encyonopsis microcephala	ENCM	
Eolimna minima	EOMI	
Fragilaria brevistriata var. inflata	FBIN	
Fragilaria capucina var. perminuta	FCPE	
Fragilaria capucina var. rumpens	FCRP	
Fragilaria capucina var. vaucheriae	FCVA	
Fragilaria construens	FCCR	
Fragilaria martyi	FMAR	
Fragilaria nanana	FNAN	
Fragilaria ulna var. acus	FUAC	
Gomphonema minusculum	GMIS	
Gomphonema olivaceum var. olivaceum	GOLI	
Gomphonema pala	GOPA	
Gomphonema parvulius	GPVL	
Gomphonema parvulum var. parvulum fo. parvulum	GPAR	YES
Gyrosigma attenuatum	GYAT	YES
Gyrosigma nodiferum	GNOD	
Meridion circulare var. circulare	MCIR	
Navicula associata	NXAS	
Navicula germainii	NGER	
Navicula perminuta	NPNU	
Navicula reichardtiana var. reichardtiana	NRCH	
Nitzschia archibaldii	NIAR	
Nitzschia recta	NREC	
Planothidium frequentissimum	PLFR	

Staurosirella pinnata	newSTPN	
Surirella brebissonii var. kuetzingii	SBKU	

*Suppl Table 3.4. List of **non-corresponding diatom species** identified from biofilm through HTS only (rbcL reference database Diat.barcode v9) from River Wertach (n = 5). New genus when no species of this genus was found in light microscopy. Proof by scanning electronic microscopy (SEM; Goos 2021) is marked with yes in field "SEM detection", if taxon is confirmed.*

V9 species (original HTS name)	HTS diatom taxon found only by rbcL	SEM detection
Achnanthidium delmontii	Achnanthidium delmontii	yes
Achnanthidium eutrophilum	Achnanthidium eutrophilum	
Amphora indistincta	Amphora indistincta	
Halamphora veneta	Amphora veneta Kützing	
Cocconeis placentula	Cocconeis placentula var. placentula	
Cyclotella atomus	Cyclotella atomus	
Cyclotella cryptica	Cyclotella cryptica	
Cyclotella distinguenda	Cyclotella distinguenda var. distinguenda	
Cyclotella meneghiniana	Cyclotella meneghiniana	
Surirella elliptica	Cymatopleura elliptica var. elliptica	
Surirella solea	Cymatopleura solea var. solea	
Cymbella excisa	Cymbella excisa var. excisa	
Cymbella lanceolata	Cymbella lanceolata var. lanceolata	
Cymbella neocistula	Cymbella neocistula var. neocistula	
Cymbella tumida	Cymbella tumida	
Diatoma tenuis	Diatoma tenuis	
Diploneis subovalis	Diploneis subovalis	
Discostella woltereckii	Discostella woltereckii	
Ellerbeckia sp.	Ellerbeckia spec	
Encyonema ventricosum	Encyonema ventricosum	YES
Craticula subminuscula	Eolimna subminuscula	
Fistulifera saprophila	Fistulifera saprophila	YES
Fragilaria gracilis	Fragilaria gracilis	
Frustulia vulgaris	Frustulia vulgaris	
Gomphonella olivacea	Gomphonella olivacea	
Gomphonema acuminatum	Gomphonema acuminatum	
Gomphonema micropus	Gomphonema micropus var. micropus	
Gomphonema saprophilum	Gomphonema parvulumvar. parvulumfo. saprophilum Lange-Bert. & Reichardt	YES
Gomphonema pumilum var. pumilum	Gomphonema pumilum	
Gomphonema pumilum var. rigidum	Gomphonema pumilum var. rigidum	
Gyrosigma sciotense	Gyrosigma sciotense	YES
Hippodonta capitata	Hippodonta capitata	
Iconella unclassified	Iconella sp.	
Karayevia ploenensis var. gessneri	Karayevia ploenensis var. gessneri	

V9 species (original HTS name)	HTS diatom taxon found only by rbcL	SEM detection
Luticola goeppertiana	Luticola goeppertiana	
Navicula rostellata	Navicula rostellata var. elongata	
Navicula trivialis	Navicula trivialis var. trivialis	
Navicula veneta	Navicula veneta	
Nitzschia acicularis	Nitzschia acicularis	
Nitzschia capitellata	Nitzschia capitellata	
Nitzschia denticula	Nitzschia denticula	
Nitzschia draveillensis	Nitzschia draveillensis	
Nitzschia inconspicua	Nitzschia inconspicua	
Nitzschia sigmoidea	Nitzschia sigmoidea	
Nitzschia acicularoides	#N/A	
Nitzschia supralitorea	Nitzschia supralitorea	
Parlibellus protracta	Parlibellus protracta	
Planothidium victori	#N/A	
Pseudostaurosira brevistriata	Pseudostaurosira brevistriata	
Sellaphora nigri	Sellaphora nigri	
Staurosira construens	Staurosira construens	
Thalassiosira pseudonana	Thalassiosira pseudonana	
Conticribra weissflogii	Thalassiosira weissflogii	
Tryblionella sp.	Tryblionella spec	

7.4 River Adige, Italy

Suppl. Table 4.3. List of cyanobacteria species and genera from biofilm identified through HTS (16S rDNA SILVA reference database) from River Adige (n = 2)

ID-REBECCA	Taxon_REBECCA	Genus_Rebecca	genus_16S	species_16S
aerophytic2	Chroococcidiopsis	Chroococcidiopsis	Chroococcidiopsis PCC-6712	NA
biofilm_new6	Phormidesmis	Phormidesmis	Phormidesmis ANT.L52.6	NA
biofilm_new6	Phormidesmis	Phormidesmis	Phormidesmis ANT.LACV5.1	NA
biofilm_new7	Wilmottia	Wilmottia	Wilmottia Ant-Ph58	NA
marin2	Aliterella	Aliterella	Aliterella	NA
new_16S_cyano_family10	Pseudanabaenaceae		NA	NA
new_16S_cyano_family12	Xenococcaceae		NA	NA
new_16S_cyano_family4	Leptolyngbyaceae		NA	NA
new_16S_cyano_family7	Nostocaceae		NA	NA
new_16S_cyano_family9	Phormidiaceae		NA	NA
R1580	Leptolyngbya sp.	Leptolyngbya	Leptolyngbya ANT.L52.2	NA
R1606	Phormidium sp.	Phormidium	Phormidium CYN64	NA
R1618	Planktothrix sp.	Planktothrix	Planktothrix NIVA-CYA 15	NA
R1623	Pseudanabaena sp.	Pseudanabaena	Pseudanabaena PCC-6802	NA
R1623	Pseudanabaena sp.	Pseudanabaena	Pseudanabaena PCC-7429	frigida
R1637	Chamaesiphon sp.	Chamaesiphon	Chamaesiphon PCC-7430	NA
R1637	Chamaesiphon sp.	Chamaesiphon	Chamaesiphon PCC-6605	minutus
R1637	Chamaesiphon sp.	Chamaesiphon	Chamaesiphon PCC-7430	subglobosus
R1948	Cyanothece sp.	Cyanothece	Cyanothece PCC 7425	NA
R2006	Pleurocapsa sp.	Pleurocapsa	Pleurocapsa PCC-7319	NA
R2710	Calothrix sp.	Calothrix	Calothrix KVSF5	NA
R2826	Tychonema sp.	Tychonema	Tychonema CCAP 1459-11B	NA

Suppl. Table 4.4. List of corresponding diatom species from biofilm identified through microscopy and through HTS (rbcL reference database R-Syst::diatom, 18S rDNA SILVA reference database) from River Adige (n = 2)

Locus	V9 species	HTS TAXON_R_Diatom	LM Validcode
rbcL	Achnanthidium delmontii	Achnanthidium delmontii	newADEL
18S, rbcL	Achnanthidium minutissimum	Achnanthidium minutissimum	ADMI
rbcL	Achnanthidium pyrenaicum	Achnanthidium pyrenaicum	ADPT
18S, rbcL	Amphora pediculus	Amphora pediculus	APED
rbcL	Craticula subminuscula	Eolimna subminuscula	ESBM
rbcL	Denticula tenuis	Denticula tenuis	DTEN
18S, rbcL	Encyonema silesiacum	Encyonema silesiacum	ESLE
rbcL	Encyonema ventricosum	Encyonema ventricosum	ENVE
18S, rbcL	Fistulifera saprophila	Fistulifera saprophila	FSAP
rbcL	Gomphonema minutum	Gomphonema minutum fo. minutum	GMIN
rbcL	Mayamaea permitis	Mayamaea atomus var. permitis	MAPE
rbcL	Navicula capitatoradiata	Navicula capitatoradiata	NCPR
18S, rbcL	Navicula cryptotenella	Navicula cryptotenella	NCTE
18S, rbcL	Navicula gregaria	Navicula gregaria	NGRE
rbcL	Navicula lanceolata	Navicula lanceolata	NLAN
18S, rbcL	Nitzschia fonticola	Nitzschia fonticola	NFON
18S, rbcL	Nitzschia paleacea	Nitzschia paleacea	NPAE
rbcL	Nitzschia unclassified	Nitzschia spec	NITZ
18S, rbcL	Reimeria sinuata	Reimeria sinuata	RSIN
rbcL	Rhoicosphenia abbreviata	Rhoicosphenia abbreviata	RABB

Suppl. Table 4.5. List of non-corresponding diatom species from microscopy to HTS (18S rDNA SILVA reference database) from River Adige (n = 2).

Taxon_validcode	LM Validcode
Achnanthidium atomoides	ADAM
Achnanthidium lineare	ACLI
Achnanthidium microcephalum	ADMC
Cocconeis placentula var. euglypta	CPLE
Cocconeis placentula var. lineata	CPLI
Eolimna minima	EOMI
Gomphonema pumilum var. elegans	GPEL
Navicula reichardtiana var. reichardtiana	NRCH
Nitzschia archibaldii	NIAR
Nitzschia dissipata var. dissipata	NDIS
Nitzschia sociabilis	NSOC
Reimeria uniseriata	RUNI
Simonsenia delognei	SIDE

Suppl. Table 4.6. List of non-corresponding diatom species from HTS (18S rDNA SILVA reference database) to microscopy to from River Adige (n = 2).

HTS TAXON_R_Diatom	Validcode
Achnanthidium eutrophilum	ADEU
Amphora ovalis	AOVA
Cocconeis pediculus	CPED
Cocconeis placentula var. placentula	CPLA
Cymatopleura elliptica var. elliptica	CELL
Cymbella compacta	CCMP
Cymbella excisa var. excisa	CAEX
Diatoma moniliformis	DMON
Diatoma vulgaris	DVUL
Didymosphenia geminata mor. geminata	DGEM
Discostella woltereckii	DWOL
Encyonema minutum	ENMI
Encyonema prostratum	EPRO
Gomphonella olivacea	GLOV
Gomphonema pumilum	GPUM
Gomphonema parvulum var. parvulum fo. saprophilum Lange-Bert. & Reichardt	GPAR
Gyrosigma sciotense	GSCI
Melosira varians	MVAR
Navicula antonii	NANT
Navicula tripunctata	NTPT
Nitzschia costei	new NCOS
Nitzschia dissipata var. media	NDME
Nitzschia linearis var. linearis	NLIN
Nitzschia palea	NPAL
Nitzschia sigmoidea	NSIO
Pseudostaurosira brevistriata	PSBR
Sellaphora nigri	new SNIG
Staurosira construens	SCON
Ulnaria ulna	UULN

7.5 River Soca, Slovenia

*Suppl Table 5.1. List of **cyanobacteria and soft algae taxons** from **biofilm** identified through microscopy from Soča River (n = 3). Frequency in classes 1 to 5, where 1 is equally very rare.*

Sampling site	Spodnja Trenta	Kamno	Solkanski jez
Taxon\date of sampling	30.08.2019	30.08.2019	30.08.2019
Homoeothrix varians	1		
Phormidium autumnale	1		
Phormidium sp.		1	1
Cosmarium sp.			1
Oedogonium sp.			1
Spirogyra sp.			1

*Suppl Table 5.2. List of **cyanobacteria** from **biofilm** identified through HTS (16S rDNA SILVA reference database) from Soča River (n = 3)*

ID REBECCA	Taxon_REBECCA	Family_16S	Genus_16S	Species_16S
aerophytic2	Chroococcidiopsis	Xenococcaceae	Chroococcidiopsis PCC-6712	NA
biofilm_new6	Phormidesmis	Phormidesmiaceae	Phormidesmis ANT.LACV5.1	NA
fresh_new4	Microseira	Cyanobacteriales Incertae Sedis	Microseira Carmichael-Alabama	NA
marin2	Aliterella	Chroococcidiopsaceae	Aliterella	NA
new_16S_cyano_family10	Pseudanabaenaceae	Pseudanabaenaceae	NA	NA
new_16S_cyano_family4	Leptolyngbyaceae	Leptolyngbyaceae	LB3-76	NA
new_16S_cyano_family4	Leptolyngbyaceae	Leptolyngbyaceae	NA	NA
new_16S_cyano_family7	Nostocaceae	Nostocaceae	NA	NA
new_16S_cyano_family9	Phormidiaceae	Phormidiaceae	NA	NA
R0888	Gloeocapsa sp.	Gloeocapsaceae	Gleocapsa	NA
R1427	Aphanothece clathrata	Cyanobiaceae	Cyanobium PCC-6307	NA
R1478	Merismopedia sp.	Cyanobacteriaceae	Merismopedia AICB1015	NA
R1496	Microcystis sp.	Microcystaceae	Microcystis PCC-7914	NA
R1518	Synechococcus sp.	Pseudanabaenaceae	Synechococcus PCC-7502	NA
R1580	Leptolyngbya sp.	Leptolyngbyaceae	Leptolyngbya FYG	NA
R1580	Leptolyngbya sp.	Leptolyngbyaceae	Leptolyngbya SAG 2411	NA
R1580	Leptolyngbya sp.	Unknown Family	Leptolyngbya ANT.L52.2	NA
R1606	Phormidium sp.	Unknown Family	Phormidium CYN64	NA
R1618	Planktothrix sp.	Phormidiaceae	Planktothrix NIVA-CYA 15	NA
R1623	Pseudanabaena sp.	Pseudanabaenaceae	Pseudanabaena PCC-6802	NA
R1623	Pseudanabaena sp.	Pseudanabaenaceae	Pseudanabaena PCC-7429	foetida/limnetica
R1623	Pseudanabaena sp.	Pseudanabaenaceae	Pseudanabaena PCC-7429	frigida
R1637	Chamaesiphon sp.	Leptolyngbyaceae	Chamaesiphon PCC-7430	NA
R2006	Pleurocapsa sp.	Xenococcaceae	Pleurocapsa PCC-7319	NA
R2090	Geitlerinema sp.	Cyanobacteriaceae	Geitlerinema LD9	NA
R2302	Cyanobium sp.	Cyanobiaceae	Cyanobium PCC-6307	gracile
R2302	Cyanobium sp.	Cyanobiaceae	Cyanobium PCC-6307	NA
R2710	Calothrix sp.	Unknown Family	Calothrix KVSF5	NA
R2826	Tychonema sp.	Phormidiaceae	Tychonema CCAP 1459-11B	bornetii/

				bourrellyi/ tenue
R2826	Tychonema sp.	Phormidiaceae	Tychonema CCAP 1459-11B	NA

*Suppl Table 5.3. List of **soft algae** from **biofilm** identified through HTS (18S rDNA SILVA reference database) from Soča River (n = 3)*

ID REBECCA	Taxon_REBECCA	Family_18S	Genus_18S	Species_18Sraw
new18R103	Unruhdinium penardii	Kryptoperidiniaceae	Unruhdinium	Unruhdinium_penardii
new18R108	Esoptrodinium sp.	Tovelliaceae	Esoptrodinium	Esoptrodinium_sp.
new18R12	Asulcocephalium miricentonis	Suessiaceae	Asulcocephalium	Asulcocephalium_miricentonis
new18R15	Chlorochytrium lemnae	Chrysophyceae_Clade-B2	Chrysochaete	Chrysochaete_britannica
new18R2	Eustigmatophyceae	Eustigmatophyceae_XX	NA	NA
new18R28	Heribaudiella fluviatilis	Phaeophyceae_XX	Heribaudiella	Heribaudiella_fluviatilis
new18R3	Trebouxiophyceae	Chlorellales_X	NA	NA
new18R36	Mougeotia scalaris	Zygnemophyceae_XX	Mougeotia	Mougeotia_scalaris
new18R37	Mychonastes sp.	Sphaeropleales_X	Mychonastes	Mychonastes_sp.
new18R37	Mychonastes sp.	Sphaeropleales_X	Mychonastes	NA
new18R4	Ulvophyceae	Cladophorales_X	Cladophora	Cladophora_glomerata
new18R4	Ulvophyceae	NA	NA	NA
new18R4	Ulvophyceae	Ulotrichales_X	NA	NA
new18R4	Ulvophyceae	Ulvales-relatives_X	Acrochaete	NA
new18R4	Ulvophyceae	Ulvales-relatives_X	NA	NA
new18R40	Aphanochaete sp.	Chaetophorales_X	Aphanochaete	NA
new18R5	Chaetophorales	Chaetophorales_X	NA	NA
new18R56	Stigeoclonium sp.	Chaetophorales_X	Stigeoclonium	NA
new18R66	Oocystis nephrocytioides	Chlorellales_X	Oocystis	Oocystis_nephrocytioides
new18R77	Poteriospumella_lacustris	Chrysophyceae_Clade-C	Poteriospumella	Poteriospumella_lacustris
new18R86	Scenedesmus obliquus	Sphaeropleales_X	Scenedesmus	Scenedesmus_obliquus

*Suppl Table 5.4. List of **diatom species** from biofilm identified through microscopy and through HTS (rbcL reference database R-Syst::diatom, 18S rDNA SILVA reference database) from Soča River (n = 3)*

Locus	HTS	Validcode	Microscopy
		AAEQ	Amphora aequalis
rbcl	Achnanthidium unclassified	ACHD	Achnanthidium sp.
		ADMF	Achnanthidium affine
rcbL, 18S	Achnanthidium minutissimum	ADMI	Achnanthidium minutissimum
rcbL	Adlafia minuscula	ADMS	Adlafia minuscula
rcbL	Achnanthidium pyrenaicum	ADPT	Achnanthidium pyrenaicum
rcbL, 18S	Amphora unclassified	AMPH	Amphora sp.
rbcl	Aneumastus unclassified	ANEU	
		ANSS	Aneumastus stroesei
rcbL	Amphora pediculus	APED	Amphora pediculus
rbcL	Cymbella excisa	CAEX	

		CAFF	Cymbella affinis
rbcL, 18S	Caloneis unclassified	CALO	
18S	Cymbella affinis	CATG	
		CCYM	Cymbella cymbiformis
rcbL	Cyclotella distinguenda	CDTG	
rbcl	Caloneis fontinalis	CFON	
rbcL	Cymbella lanceolata	CLAN	
		CLCT	Caloneis lancettula
rcbL	Cyclotella meneghiniana	CMEN	
18S	Cocconeis sp.	COCM	
		COPL	Cocconeis pseudolineata
rbcL	Cocconeis pediculus	CPED	Cocconeis pediculus
rbcl, 18S	Cocconeis placentula	CPLA	Cocconeis cf. placentula
		CPLE	Cocconeis euglypta
		CPLK	Cocconeis placentula
18S	Cymbella sp.	CYMB	
rbcL, 18S	Didymosphenia geminata	DGEM	
		DGEM	Didymosphenia geminata
		DIAT	Odontidium sp.
rbcL	Discostella nipponica	DNIP	
rcbL	Denticula tenuis	DTEN	Denticula tenuis
		DVUL	Diatoma vulgaris
rbcL, 18S	Diatoma vulgaris	DVUL	Diatoma vulgaris
rbcL	Discostella woltereckii	DWOL	
rbcL, 18S	Encyonema caespitosum	ECAE	
rbcl	Encyonopsis minuta	ECPM	
rcbL	Ellerbeckia sp.	ELLE	
		ENCM	Cymbella microcephala
18S	Encyonopsis sp.	ENCP	
rcbL	Encyonema unclassified	ENCY	
rcbL	Encyonema minutum	ENMI	Encyonema minutum
		ENVE	Encyonema ventricosum
rbcL, 18S	Encyonema silesiacum	ESLE	Encyonema silesiacum
		ESOR	Epithemia sorex
rcbL	Encyonopsis subminuta	ESUM	
18S	Epithemia turgida	ETUR	
		EULA	Eucocconeis laevis
		FCAP	Fragilaria cf. capucina
		FCPE	Fragilaria perminuta
		FCVA	Fragilaria vaucheriae
rbcL	Fragilaria gracilis	FGRA	
rbcL	Fragilaria perminuta	FPEM	
rbcl	Fragilaria acus/radians complex	FRAD	
rcbL	Fragilaria unclassified	FRAG	
rbcL, 18S	Fragilaria sp.	FRAS	
rcbL	Fistulifera saprophila	FSAP	

		FUAC	Fragilaria ulna var. acus (
		GACU	Gomphonema acuminatum
rbcL	Gomphonema affine	GAFF	
rcbL	Geissleria decussis	GDEC	Geissleria decussis
rbcL	Gomphonema minutum	GMIN	Gomphonema minutum
		GOMP	Gomphonema sp.
		GPSA	Gomphonema pseudoaugur
rcbL	Gomphonema pumilum var. pumilum	GPUM	Gomphonema pumilum
rcbL	Gomphonema tergestinum	GTER	Gomphonema tergestinum
rcbL	Iconella linearis	ICON	
rcbL	Mayamaea permitis	MAPE	
rbcL, 18S	Melosira varians	MVAR	Melosira varians
		NACI	Nitzschia acicularis
rcbL	Navicula antonii	NANT	Navicula antonii
18S	Navicula sp.	NAVI	Navicula sp.
rcbL	Navicula capitatoradiata	NCPR	Navicula capitatoradiata
		NCRY	Navicula cryptocephala
rbcL, 18S	Navicula cryptotenella	NCTE	Navicula cryptotenella
		NCTE	Navicula cryptotenella
rbcL	Nitzschia dissipata	NDIS	Nitzschia dissipata ssp. dissipata
rcbL	Nitzschia dissipata var. media	NDME	
rbcL	Achnanthidium delmontii	newADEL	Achnanthidium delmontii
rcbL	Nitzschia soratensis	newNSOR	
		newSTPN	Staurosirella pinnata
rcbL,18S	Nitzschia fonticola	NFON	Nitzschia fonticola
		NGRE	Navicula gregaria
rcbL	Nitzschia pusilla	NIPU	
rcbL, 18S	Nitzschia unclassified	NITZ	Nitzschia sp.
rcbL, 18S	Nitzschia linearis	NLIN	Nitzschia linearis
		NPAE	Nitzschia paleacea
rbcL, 18S	Nitzschia palea	NPAL	Nitzschia palea var. palea
18S	Navicula radiosa	NRAD	
		NSPD	Navicula splendicula
rcbL	Navicula tripunctata	NTPT	
		PBIO	Psammothidium bioretii
rcbL	Psammothidium helveticum	PHEL	
rcbL	Lindavia radiosa	PRAD	
		PSMT	Psammothidium sp.
		PTDU	Planothidium dubium
rbcl, 18S	Planothidium lanceolatum	PTLA	Planothidium lanceolatum
		PTSA	Platessa sp.
		RABB	Rhoicosphenia abbreviata
rcbL	Reimeria sinuata	RSIN	Reimeria sinuata
rcbL	Staurosira construens	SCON	
		SSVE	Staurosira venter
		SUMI	Surirella minuta

rcbL	Surirella unclassified	SURI	
18S	Synedra sp.	SYNS	
		TANG	Tryblionella angustata
rbcL	Ulnaria unclassified	ULNA	
rbcL, 18S	Ulnaria ulna	UULN	

*Suppl Table 5.5. List of **diatom species** in comparison from HTS to microscopy for Soča River (n = 3).*

	TAXON_HTS	Validcode	Taxon_Microscopy
Kamno	Achnanthidium minutissimum	ADMI	Achnanthidium minutissimum
	Achnanthidium pyrenaicum	ADPT	Achnanthidium pyrenaicum
	Achnanthidium delmontii	newADEL	
	Caloneis spec	CALO	
	Cocconeis pediculus	CPED	Cocconeis pediculus
	Cyclotella distinguenda var. distinguenda	CDTG	
	Cymbella excisa var. excisa	CAEX	
	Cymbella lanceolata var. lanceolata	CLAN	
	Denticula tenuis	DTEN	Denticula tenuis
	Diatoma vulgaris	DVUL	Diatoma vulgaris
	Didymosphenia geminata mor. geminata	DGEM	
	Discostella nipponica	DNIP	
	Discostella woltereckii	DWOL	
	Encyonema caespitosum	ECAE	
	Encyonema spec	ENCY	
	Encyonema silesiacum	ESLE	Encyonema silesiacum
	Encyonopsis subminuta	ESUM	
	Fragilaria gracilis	FGRA	
	Fragilaria perminuta	FPEM	
	Fragilaria spec	FRAG	
	Fragilaria species	FRAS	
	Gomphonema affine	GAFF	
	Gomphonema minutum fo. minutum	GMIN	Gomphonema minutum fo. minutum
	Melosira varians	MVAR	
	Navicula capitatoradiata	NCPR	
	Navicula cryptotenella	NCTE	Navicula cryptotenella
	Navicula tripunctata	NTPT	
	Nitzschia dissipata var. dissipata	NDIS	Nitzschia dissipata var. dissipata
	Nitzschia fonticola	NFON	Nitzschia fonticola
	Nitzschia linearis var. linearis	NLIN	Nitzschia linearis var. linearis
	Nitzschia palea	NPAL	Nitzschia palea
	Staurosira construens	SCON	
	Ulnaria spec	ULNA	
	Ulnaria ulna	UULN	

Solkanski jez	Achnanthidium spec	ACHD	Achnanthidium spec
	Achnanthidium minutissimum	ADMI	Achnanthidium minutissimum
	Achnanthidium pyrenaicum	ADPT	Achnanthidium pyrenaicum
	Adlafia minuscula	ADMS	
	Amphora spec	AMPH	Amphora spec
	Amphora pediculus	APED	Amphora pediculus
	Aneumastus spec	ANEU	
	Caloneis fontinalis	CFON	
	Cocconeis placentula var. placentula	CPLA	Cocconeis placentula var. placentula
	Cymbella excisa var. excisa	CAEX	
	Denticula tenuis	DTEN	Denticula tenuis
	Encyonema spec	ENCY	
	Encyonopsis minuta	ECPM	
	Fistulifera saprophila	FSAP	
	Fragilaria radians	FRAD	
	Gomphonema pumilum	GPUM	Gomphonema pumilum
	Gomphonema tergestinum	GTER	Gomphonema tergestinum
	Navicula antonii	NANT	Navicula antonii
	Nitzschia dissipata var. media	NDME	
	Nitzschia soratensis	newNSOR	
	Nitzschia fonticola	NFON	Nitzschia fonticola
	Nitzschia spec	NITZ	
	Planothidium lanceolatum	PTLA	
	Psammothidium helveticum	PHEL	
	Reimeria sinuata	RSIN	
	Staurosira construens	SCON	
	Ulnaria ulna	UULN	
Spodnja Trenta	Achnanthidium minutissimum	ADMI	Achnanthidium minutissimum
	Achnanthidium pyrenaicum	ADPT	Achnanthidium pyrenaicum
	Adlafia minuscula	ADMS	
	Amphora spec	AMPH	
	Amphora pediculus	APED	Amphora pediculus
	Caloneis spec	CALO	
	Cocconeis placentula var. placentula	CPLA	
	Cyclotella distinguenda var. distinguenda	CDTG	
	Cyclotella meneghiniana	CMEN	
	Denticula tenuis	DTEN	Denticula tenuis
	Ellerbeckia spec	ELLE	
	Encyonema spec	ENCY	
	Encyonema minutum	ENMI	
	Encyonopsis subminuta	ESUM	
	Fistulifera saprophila	FSAP	
	Fragilaria spec	FRAG	
	Geissleria decussis	GDEC	
	Gomphonema pumilum	GPUM	Gomphonema pumilum

	Gomphonema tergestinum	GTER	Gomphonema tergestinum
	Iconella sp.	ICON	
	Mayamaea atomus var. permitis	MAPE	
	Navicula antonii	NANT	Navicula antonii
	Navicula capitatoradiata	NCPR	
	Navicula cryptotenella	NCTE	
	Navicula tripunctata	NTPT	
	Nitzschia dissipata var. media	NDME	
	Nitzschia soratensis	newNSOR	
	Nitzschia fonticola	NFON	Nitzschia fonticola
	Nitzschia pusilla	NIPU	
	Nitzschia spec	NITZ	
	Nitzschia linearis var. linearis	NLIN	
	Nitzschia palea	NPAL	Nitzschia palea
	Planothidium lanceolatum	PTLA	Planothidium lanceolatum
	Psammothidium helveticum	PHEL	
	Puncticulata radiosa	PRAD	
	Reimeria sinuata	RSIN	Reimeria sinuata
	Staurosira construens	SCON	
	Surirella spec	SURI	

*Suppl Table 5.6. List of **diatom species** in comparison from microscopy to HTS for Soča River (n = 3).*

Samplig site	Taxon_Microscopy	Validcode	detectable by rcbl	Taxon_HTS
Spodnja Trenta	Achnanthidium minutissima var. affinis	ADMF		
	Achnanthidium minutissimum	ADMI	yes	Achnanthidium minutissimum
	Achnanthidium pyrenaicum	ADPT	yes	Achnanthidium pyrenaicum
	Aneumastus stroesei	ANSS		
	Amphora pediculus	APED	yes	Amphora pediculus
	Cymbella affinis var. affinis	CAFF		
	Caloneis lancettula	CLCT		
	Cocconeis pseudolineata	COPL		
	Cocconeis placentula var. euglypta	CPLE		
	Denticula tenuis	DTEN	yes	Denticula tenuis
	Diatoma vulgaris	DVUL	yes	
	Encyonema ventricosum	ENVE	yes	
	Eucocconeis laevis	EULA		
	Gomphonema spec	GOMP	yes	
	Gomphonema pumilum	GPUM	yes	Gomphonema pumilum
	Gomphonema tergestinum	GTER	yes	Gomphonema tergestinum
	Navicula antonii	NANT	yes	Navicula antonii
	Nitzschia dissipata var. dissipata	NDIS	yes	
	Achnanthidium pseudolineare	newAPSL		
	Nitzschia fonticola	NFON	yes	Nitzschia fonticola
	Nitzschia palea	NPAL	yes	Nitzschia palea
	Navicula splendicula	NSPD		

	Psammothidium bioretii	PBIO		
	Psammothidium spec	PSMT	yes	
	Planothidium dubium	PTDU		
	Planothidium lanceolatum	PTLA	yes	Planothidium lanceolatum
	Platessa sp.	PTSA		
	Reimeria sinuata	RSIN	yes	Reimeria sinuata
Kamno	Amphora aequalis	AAEQ		
	Achnanthidium spec	ACHD	yes	
	Achnanthidium minutissimum	ADMI	yes	Achnanthidium minutissimum
	Adlafia minuscula	ADMS	yes	
	Achnanthidium pyrenaicum	ADPT	yes	Achnanthidium pyrenaicum
	Amphora pediculus	APED	yes	
	Cocconeis pseudolineata	COPL		
	Cocconeis pediculus	CPED	yes	Cocconeis pediculus
	Cocconeis placentula var. placentula	CPLA	yes	
	Cocconeis placentula var. euglypta	CPLE		
	Diatoma spec	DIAT		
	Denticula tenuis	DTEN	yes	Denticula tenuis
	Diatoma vulgaris	DVUL	yes	Diatoma vulgaris
	Encyonema minutum	ENMI	yes	
	Encyonema ventricosum	ENVE	yes	
	Encyonema silesiacum	ESLE	yes	Encyonema silesiacum
	Fragilaria capucina var. vaucheriae	FCVA		
	Geissleria decussis	GDEC	yes	
	Gomphonema minutum fo. minutum	GMIN	yes	Gomphonema minutum fo. minutum
	Gomphonema spec	GOMP	yes	
	Gomphonema tergestinum	GTER	yes	
	Navicula antonii	NANT	yes	
	Navicula spec	NAVI	yes	
	Navicula cryptotenella	NCTE	yes	Navicula cryptotenella
	Nitzschia dissipata var. dissipata	NDIS	yes	Nitzschia dissipata var. dissipata
	Achnanthidium pseudolineare	newAPSL		
	Staurosirella pinnata	newSTPN		
	Nitzschia fonticola	NFON	yes	Nitzschia fonticola
	Navicula gregaria	NGRE	yes	
	Nitzschia spec	NITZ	yes	
	Nitzschia linearis var. linearis	NLIN	yes	Nitzschia linearis var. linearis
	Nitzschia paleacea	NPAE	yes	
	Nitzschia palea	NPAL	yes	Nitzschia palea
	Navicula splendicula	NSPD		
	Planothidium dubium	PTDU		
	Rhoicosphenia abbreviata	RABB	yes	
	Reimeria sinuata	RSIN	yes	
	Staurosira venter	SSVE	yes	
	Achnanthidium spec	ACHD	yes	Achnanthidium spec

Solkanski jez	Achnanthidium minutissima var. affinis	ADMF		
	Achnanthidium minutissimum	ADMI	yes	Achnanthidium minutissimum
	Achnanthidium pyrenaicum	ADPT	yes	Achnanthidium pyrenaicum
	Amphora spec	AMPH	yes	Amphora spec
	Amphora pediculus	APED	yes	Amphora pediculus
	Cymbella affinis var. affinis	CAFF		
	Cymbella cymbiformis	CCYM	yes	
	Caloneis lancettula	CLCT		
	Cocconeis pseudolineata	COPL		
	Cocconeis pediculus	CPED	yes	
	Cocconeis placentula var. placentula	CPLA	yes	Cocconeis placentula var. placentula
	Cocconeis placentula var. euglypta	CPLE		
	Cocconeis placentula var. klinoraphis	CPLK		
	Didymosphenia geminata mor. geminata	DGEM	yes	
	Denticula tenuis	DTEN	yes	Denticula tenuis
	Diatoma vulgaris	DVUL	yes	
	Encyonopsis microcephala	ENCM	yes	
	Encyonema minutum	ENMI	yes	
	Encyonema ventricosum	ENVE	yes	
	Epithemia sorex	ESOR	yes	
	Fragilaria capucina var. capucina	FCAP		
	Fragilaria capucina var. perminuta	FCPE		
	Fragilaria ulna var. acus	FUAC		
	Gomphonema acuminatum	GACU	yes	
	Gomphonema spec	GOMP	yes	
	Gomphonema pseudoaugur	GPSA		
	Gomphonema pumilum	GPUM	yes	Gomphonema pumilum
	Gomphonema tergestinum	GTER	yes	Gomphonema tergestinum
	Melosira varians	MVAR	yes	
	Nitzschia acicularis	NACI	yes	
	Navicula antonii	NANT	yes	Navicula antonii
	Navicula capitatoradiata	NCPR	yes	
	Navicula cryptocephala	NCRY	yes	
	Navicula cryptotenella	NCTE	yes	
	Nitzschia dissipata var. dissipata	NDIS	yes	
	Achnanthidium delmontii	newADEL	yes	
	Achnanthidium pseudolineare	newAPSL		
	Nitzschia fonticola	NFON	yes	Nitzschia fonticola
	Surirella minuta	SUMI		
	Tryblionella angustata	TANG		

Suppl Table 5.6. List of fish obtained by HTS method in sampling site Kamno in Soča River (2019)

Taxon	Signal
Cottidae	302988
Cyprinidae	5278
Barbus ciscaucasicus	1610
Phoxinus phoxinus	11130
Squalius cephalus	141
Salmoninae	103447
Oncorhynchus mykiss	374168
Salmo	20797
Salvelinus	420
Thymallus thymallus	7495

Suppl Table 5.7. List of fish obtained by traditional method in sampling site Kamno in Soča River (2017)

Taxon	Abundance	Biomass [g]
Salmo marmorata	148	565,2
Cottus gobio	609	3396,7
Thymallus thymallus	1	2,6
Oncorhynchus mykiss	6	5240