ecologies

MDPI

Article

Bridging the Gap: Integrating DNA Metabarcoding and Microscopy to Assess Benthic Diatoms and Their Environmental Drivers in a Mountain Lake of the Alps

Ulrike Obertegger [1,2,*], Cristina Cappelletti [3], Saúl Blanco [4], Massimo Pindo [1], Marco Bertoli [5], Elisabetta Pizzul [5], Paolo Pastorino [6], Marino Prearo [6] and Francesca Ciutti [3]

[1] Research and Innovation Centre, Fondazione Edmund Mach, 38098 San Michele all'Adige, Italy; massimo.pindo@fmach.it
[2] National Biodiversity Future Center—NBFC, Piazza Marina 61, 90133 Palermo, Italy
[3] Technological Transfer Centre, Fondazione Edmund Mach, 38098 San Michele all'Adige, Italy; cristina.cappelletti@fmach.it (C.C.); francesca.ciutti@fmach.it (F.C.)
[4] Diatom Lab, IMARENABIO, La Serna 58, 24007 Leon, Spain; sblal@unileon.es
[5] Department of Life Sciences, University of Trieste, Via L. Giorgieri 10, 34127 Trieste, Italy; marbertoli@units.it (M.B.); pizzul@units.it (E.P.)
[6] Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna 148, 10154 Torino, Italy; paolo.pastorino@izsto.it (P.P.); marino.prearo@izsto.it (M.P.)
* Correspondence: ulrike.obertegger@fmach.it; Tel.: +39-0461615377

Abstract

Metabarcoding is increasingly used for monitoring, but its usefulness for assessing benthic diatom diversity in mountain lakes is poorly understood, especially for Italy. We assessed benthic diatom diversity in Upper Lake Balma (2204 m asl; North-Western Alps; Italy) using microscopy and metabarcoding (amplicon sequence variants—ASVs—of the rbcL gene) during July and October 2021. We found a higher diversity with metabarcoding than with microscopy but metabarcoding missed taxa detected with microscopy and vice versa. The application of a correction factor based on biovolume to the metabarcoding dataset did not markedly improve the similarity between the methods. With metabarcoding, only 53% of ASVs were identified to the species level. Metabarcoding indicated good water quality, whereas microscopy indicated high quality. Among ecological guilds, diatoms of the guild high-profile dominated in July and October with both methods. Similarly, monthly differences linked to similar environmental variables (pH, temperature, conductivity) were indicated by multivariate ordination based on the abundance of ASVs and taxa-$_{\text{MICROSCOPY}}$, respectively. The vast occurrence of diatom taxa classified as worthy of protection emphasized the ecological value of the lake. Unless reference databases reflect diversity of the mountain lakes, we suggest the complementary use of both methods to comprehensively assess benthic diatom diversity.

Keywords: Upper Lake Balma; alpine lake; indicator species; rbcL gene; diatom red list; biovolume correction factor

Check for updates

Academic Editor: Volker Lüderitz

Received: 23 September 2026
Revised: 10 October 2026
Accepted: 17 October 2026
Published: 21 October 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

1. Introduction

Diatoms are widely used bioindicators in freshwater ecosystems as they provide valuable insights into trophic and thermal conditions [1] and indicate localized pollution and ecological changes in rivers and lowland lakes [2,3]. The European Water Framework Directive excludes high-elevation mountain lakes, resulting in limited studies of

diatom communities in these environments. Mountain lakes are sensitive indicators of environmental degradation and global climate change and deserve special attention [4,5]. Furthermore, these environments are characterized by harsh environmental conditions linked to an almost year-round ice-cover, low nutrient levels, high radiation, and low water temperatures [6]. Nevertheless, in mountain lakes a diverse benthic diatom community develops that is dominated by alpine and acidophilous taxa mainly belonging to the genera *Psammothidium* and *Eunotia* [7]. Despite studies acknowledging the high benthic diatom diversity, the diversity of diatoms in mountain lakes is under-investigated [8–10]. Not unexpectedly, recent studies describe new species in mountain lakes (e.g., [11–15]).

Generally, diatoms are identified by light microscopy, which is laborious and requires expert knowledge. Even experts face challenges in taxonomic classification [16] because of a wide range of morphologies within a group and the limited availability of distinguishing characteristics between species (e.g., [17]) and/or the occurrence of cryptic species [18]. Recently, metabarcoding of environmental DNA (eDNA) has been used as a fast and relatively cheap alternative to microscopy for monitoring purposes [19]. Both microscopy and metabarcoding have biases such as (i) the upper limit of frustule detection is much lower (500 frustules) with microscopy than with metabarcoding (>10,000 reads), (ii) dead diatoms are assessed by both methods, (iii) incomplete coverage of species in reference barcode libraries, semi-quantitative abundance assessment, and primer bias by metabarcoding [19], and (iv) the observation of yet undescribed species by both methods.

To circumvent the issue of incomplete reference libraries in metabarcoding, alternative approaches have been proposed, such as using a taxonomy-free approach that allows the computing of a molecular index directly from eDNA data [20] or direct trait assignment of 23S sequences [21] without any reference to morphotaxonomy. Additionally, applying a correction factor based on diatom biovolume was specifically introduced to reduce quantification bias in metabarcoding [21]. Although this method has proven effective in some contexts [22,23], its adoption across studies remains variable [24].

Metabarcoding of eDNA can be a valuable approach but nevertheless needs careful consideration. Summer littoral benthic diatoms from 56 alpine lakes (1300–2500 m above sea level) of the French Alps show higher values of alpha- and gamma-diversity indices and lower values of beta-diversity indices with metabarcoding of the rbcL gene than with microscopy linked to the higher detection capacity of rare taxa with metabarcoding [25]. Similarly for marine benthic diatoms and benthic diatoms of Hungarian Soda pans and rivers, metabarcoding of the rbcL gene reveals genera that remain undetected by microscopy, and authors suggest the complementary use of both approaches [22,26]. For river [23] and coastal [24] benthic diatoms, metabarcoding of the rbcL gene shows the same biodiversity at the genus but not at the species level compared to microscopy. Studies covering various aquatic environments such as Northern European lakes [27], Hungarian soda pans and rivers [23], and Eastern European rivers [28] show that the ecological status scores for the Specific Pollution Sensitivity Index (SPI/IPS) are lower with metabarcoding of the rbcL gene than with traditional microscopy.

To date, no study has compared benthic diatom diversity derived from microscopy and metabarcoding in an Italian Alpine lake. To fill this knowledge gap, we evaluated July and October benthic diatom communities using both methods from seven sampling sites in Upper Lake Balma (Northern Italy), a key site for studying environmental change in the region [29–31]. Given the ongoing, silent loss of taxonomic expertise [32], our study assesses the taxonomic gap between microscopy-based taxonomy and eDNA-based metabarcoding in this lake. We specifically compared biodiversity assessments derived from microscopy to both a biomass-corrected dataset (*sensu* [33]) and an uncorrected metabarcoding dataset to evaluate the importance of this correction step for diatom biodiversity evaluation in

mountain lakes. We used the rbcL gene as it is linked to the dedicated barcode reference library for diatoms (Diat.barcode [34]) and is widely used in metabarcoding studies on diatoms, allowing comparisons with other studies. We hypothesized differences between microscopy and metabarcoding especially at the species level because of a supposed low coverage for species from mountain lakes. Additionally, we investigated seasonal variations in diatom communities and their environmental drivers, analyzed differences in diatom ecological guilds, assessed seasonal indicator species, and evaluated the Red-List status of diatom taxa as a metric for freshwater ecological integrity [35].

2. Materials and Methods

2.1. Study Site

Upper Balma Lake (altitude: 2214 m above sea level; area: 1.82 ha; maximum depth: 2.77 m; longitude: 45°02′15.055″ N; latitude: 07°10′27.724″ E; Figure S1) is a small and shallow lake in the Cottian Alps (North-Western Alps, Italy, Piedmont). Upper Lake Balma has been studied for topics such as macrobenthic invertebrate assemblages [29], paleoecological investigations of testate amoeba and chironomid assemblages [30], and microplastic pollution [36]. The lake catchment is mainly composed of ophiolite metamorphic bedrocks. Details on the study area and physicochemical water variables can be found in [29]. Anthropogenic alterations of the lake include fish introduction (*Salvelinus fontinalis* Mitchill, 1814), grazing livestock, and microplastics [36–38].

2.2. Environmental Variables

Water samples (1 L) for nutrient analysis and physicochemical parameters were collected from seven littoral sites (Figure S1) in July and October 2021, representing snap-shots of summer and autumn, respectively. Sampling sites were chosen to fully cover the variability of littoral sites. Nitrate (μg L^{-1}), ammonium (μg L^{-1}), and phosphate (μg L^{-1}) were determined as described in [29]. Water temperature (°C), % oxygen saturation, pH (unit), and conductivity (μS cm^{-1}) were recorded using field meters (HI 9033 conductivity meter, HI 9125 pH/ORP meter, HI 9147 oximeter; Hanna Instruments Inc., Woonsocket, RI, USA).

2.3. Diatom Sampling and Microscopy

Diatoms were collected at the same seven littoral sites as for environmental variables and were taken from hard substrates (boulders, cobbles) by scraping the periphyton with a toothbrush. We acknowledge that using seven sites per month might seem limited, but it was reasonable considering the small size of the lake, and a sample size <20 is common in studies comparing metabarcoding to microscopy [22,24,39]. Samples were preserved in 70% ethanol, stored at 4 °C, and then processed in the laboratory to obtain permanent slides by cold and hot oxidation with 37% H_2O_2 and $K_2Cr_2O_7$ and addition of 1N HCl. Cleaned frustules were mounted in Naphrax to obtain permanent slides. For morphological analysis, 500 valves per sample were counted and identified to species level (microscope: Olympus BX51; magnification: 1000× DIC; imaging system: Leica DMC4500 (Heerbrugg, Switzerland) and LAS Version 4.12.0) using several guides (details in [8]). Nomenclature was identified according to Algaebase [40]. We refer to $taxa_{MIC}$ from here onwards to indicate taxa that were identified morphologically by microscopy.

2.4. eDNA Extraction, Amplicon Library Preparation, and Sequencing

For metabarcoding, eDNA was extracted from the same biofilm samples collected for microscopy using the NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) according to [41]. Amplification of the rbcL gene was carried out with primers DIAT rbcL 708F mix and DIAT rbcL R3 mix (Table S1), combining an equimolar mix at 10 μM final concentration

each [42]. For each amplification, 2 µL of diluted DNA from 5 to 20 ng μL^{-1} in a final volume of 25 µL was used. Each PCR mix was composed of 0.25 µL Q5 high-fidelity DNA polymerase, 5 µL 5× Q5 reaction buffer, 1.25 µL 10 µM forward primer, 1.25 µL 10 µM reverse primer, 0.5 µL 10 mM dNTPs and Nuclease-Free Water to 25 µL. The thermocycling conditions were initiated by a denaturation step at 98 °C for 3 min, followed by a total of 33 cycles of 98 °C denaturation for 10 s, 55 °C annealing for 30 s, 72 °C extension for 30 s, followed by a final extension at 72 °C for 10 min.

The amplification products were checked on 1.5% agarose gel and purified using the CleanNGS beads (CleanNa, Waddinxveen, The Netherlands) following the manufacturer's instructions. Subsequently, a second PCR round was used to apply dual indices and Illumina sequencing adapters Nextera XT Index Primer (Illumina, San Diego, CA, USA) by 8 PCR cycles (16S Metagenomic Sequencing Library Preparation, Illumina). The amplicon libraries were purified using the CleanNGS beads (CleanNa, Waddinxveen, The Netherlands), and the quality control was performed on a Typestation 4150 platform (Agilent Technologies, Santa Clara, CA, USA). Finally, all barcoded libraries were pooled in an equimolar way and sequenced using the V.3 kit PE300 on an Illumina®MiSeq platform (MiSeq Control Software 2.5.0.5 and Real-Time Analysis software 1.18.54.0).

2.5. Bioinformatics Pipeline

Cutadapt [43] was used to trim primers from the demultiplexed paired-end reads. We applied a custom pipeline to infer amplicon sequence variants (ASVs) from the diatom metabarcoding sequence data (available on https://github.com/fkeck/DADA2_diatoms_pipeline (accessed on 15 May 2025) [44]) using DADA2 version 1.18.0 [45]. Taxonomic assignment of ASVs was carried out with the open-access Diat.barcode reference library (Diat.barcode_rbcL_263bp_DADA2 based on Diat.barcode v12 from 10 June 2024 [34]) and a minimum bootstrap confidence of 80 for taxonomic assignment. All ASVs not assigned to diatoms and only sequences with 263 bp were retained. Sequences were translated to amino acids using MEGA10 [46] to check for stop codons (none were found). Lastly, ASVs which presented less than 8 reads in the entire database were removed to eliminate erratic ASVs.

We applied a cell biovolume correction factor (CF) to the metabarcoding dataset to consider quantification bias [33] and to investigate the importance of this approach for the assessment of benthic diatom diversity in a mountain lake. For ASVs assigned to the species level, we obtained the CF from the diat.barcode database [47] and using the biovolume-based equation [33] to obtain the CF. For ASVs with missing taxonomic assignment, we attributed the mean CF over all found taxa starting from genus level and moving to higher taxonomic levels. For example, 54 ASVs did not have a genus name but were assigned to a family, and thus we used the mean CF over all ASVs that belonged to the same family within Lake Balma. Finally, the read number was divided by this CF to obtain read counts corrected for the quantification bias in metabarcoding [33]. Therefore, the abundance of reads was lower for the CF-corrected compared to the not CF-corrected database. From here onwards, we refer to the metabarcoding dataset that was CF-corrected as *rbcL*-CF and that was not corrected as *rbcL*. When results of both *rbcL*-CF and *rbcL* showed no differences, we referred to them as results of metabarcoding in general without method discrimination. Nomenclature of the metabarcoding dataset was according to Algaebase [40]. We consider the sequences found as a fingerprint of total biodiversity, present and past because eDNA originates both from intact, viable cells (i.e., active and dormant cells), dead and broken cells, or from DNA bound to inorganic and organic particles [48].

2.6. Diatom Red List

A diatom-Red-List based approach has a high potential in describing ecological integrity of freshwater ecosystems [32]. We adopted the conservation status of diatoms according to [49] presented at the German red list centre (https://www.rote-liste-zentrum.de queried accessed on 30 June 2025).

2.7. Biological Quality Assessment

Among the many autecological metrics, the SPI [50] is one of the most frequently used diatom-based indices. Although specific diatom indices have been developed for lake monitoring (e.g., [51]), indices originally dedicated to rivers have also been used for lakes [52,53]. SPI utilizes the relative abundance of species in assemblages, along with their ecological preferences, sensitivities, or tolerances, and it is a powerful tool for inferring environmental conditions in ecosystems [54–56]. Ranges of SPI scores indicate water quality: bad (1–5), poor (5–9), moderate (9–13), good (13–17), or high quality (17–20). We used library diathor [57] to calculate SPI for microscopy and metabarcoding.

2.8. Ecological Guilds

Ecological guilds are defined by a species' capacity to utilize nutrient resources and withstand physical disturbances. Four guilds are distinguished (high-profile, low-profile, motile, planktic) and are useful to investigate the ecology of diatoms [58]. We attributed ecological guilds based on [3] and expert knowledge (*Aulacoseira granulata* was set as planktic while all other species of *Aulacoseira* were set as high-profile). The guild approach is used for a better understanding of diatoms ecology as functional aspects can provide generality and predictability of ecological patterns [59].

2.9. Data Analysis

In all analyses except for the multivariate analysis, we used the seven sampling sites within Upper Lake Balma to capture spatial variation when assessing monthly differences in benthic diatom diversity. We acknowledge that diatoms often show a high spatial variability, but we assume that seasonal differences are larger than site differences within seasons, especially considering the small size of the lake (<2 ha). For analyses on alpha-diversity, the ASV table was rarefied to an even depth specific for each primer [60]. Rarefaction of the *rbcL*-CF and *rbcL* metabarcoding datasets led to different numbers of ASVs because the read number of ASVs varied with the application of the CF. We evaluated taxonomic overlap between methods at the order, family, genus, and species levels using set analysis functions provided in libraries ggvenn [61] and VennDiagram [62]. Relative abundances were determined by calculating the percentage of reads (metabarcoding) and valves (microscopy) relative to the total count per sample.

We used the non-parametric Wilcoxon signed-rank sum test to investigate seasonal differences in environmental variables, the % mean values of ecological guilds, and the mean numbers of $taxa_{MIC}$ and ASVs. To investigate differences in the mean relative abundance of reads and SPI dependent on month (July versus October) and method (*rbcL* versus microscopy; *rbcL*-CF versus microscopy), we used non-parametric two-way ANOVA by using the aligned rank transform procedure (library ARTool [63]), which is especially useful with percent data. To assess seasonal differences regardless of method effects, non-parametric Wilcoxon signed-rank tests were conducted separately for the *rbcL* and microscopy-based taxonomic data.

We applied an indicator species analysis to find taxa specific for the months. For microscopy, we retained all taxa that occurred in at least two samples and used the library indicspecies [64] providing an indicator value for taxa. In detail, the indicator value is the

product of value A (i.e., the probability that the site belongs to the target group given the fact that the species has been found) and B (i.e., the probability of finding the species in sites belonging to the site group). For metabarcoding, we used differential gene expression analysis based on the negative binomial distribution with the library DESeq2 [65] which shows good performance with a small number of replicates [66]. We only report ASVs with a p-value < 0.01 adjusted for multiple testing and a mean abundance across all samples >100 reads for *rbcL* and >10 reads for *rbcL*-CF.

We applied non-metric multidimensional scaling (NMDS; library vegan [67]) to investigate monthly differences at the ASV and species level, respectively, using all sites. For NMDS, the ASV table was transformed by the modified centred-log ratio transform (m-clr; package metaMint [68]), which is appropriate for compositional data [69]). We used environmental fitting to link environmental variables to ordinations and used Procrustes analysis to compare NMDS ordinations of metabarcoding- and microscopy-based data.

In figures, we used colourblind-friendly colours (library RcolorBrewer [70]).

3. Results

3.1. Seasonal Differences in Environmental Variables

Among environmental variables, temperature, % dissolved oxygen saturation, and pH showed higher values ($p < 0.01$) during July than October while conductivity showed lower values during summer than autumn (Table 1). Especially nitrogen and phosphorus showed a large site variability, dampening any monthly difference (Table S2).

Table 1. Mean ± one standard deviation of environmental variables for seasons across sites; different letters indicate statistically significant differences ($p < 0.01$ **; $p < 0.001$ ***).

Variable	July	October
water temperature (°C)	11.5 ± 0.26 ^{a}	5.7 ± 0.14 ^{b***}
% DO saturation	77.1 ± 4.18 ^{a}	69.3 ± 3.49 ^{b**}
NO_3 (µg L^{-1})	2902 ± 1437	2589 ± 1650
NH_4 (µg L^{-1})	49 ± 23.7	37 ± 25.9
total phosphorus (µg L^{-1})	46.7 ± 1.31	54.8 ± 1.69
conductivity (µS cm^{-1})	11.7 ± 0.51 ^{a}	14.0 ± 0.76 ^{b***}
pH	7.6 ± 0.07 ^{a}	7.3 ±0.17 ^{b**}

3.2. Alpha Diversity of Benthic Diatoms

We observed a higher diversity with metabarcoding than with microscopy. Specifically, 1,785,235 high-quality reads were obtained across all 14 samples, resulting in 548 ASVs. In the metabarcoding dataset, unassigned ASVs accounted for 7% at the class level, 11% at the order level, 13% at the family level, 22% at the genus level, and 47% at the species level (Table S3). Consequently, correction factors (CFs) were assigned using mean values across corresponding taxonomic ranks to generate the *rbcL*-CF dataset (Table S3).

After rarefying to an even sequencing depth, 68,432 reads per sample and a total of 548 ASVs remained for *rbcL*, whereas 20,916 reads per sample and 543 ASVs remained for *rbcL*-CF. No differences in taxonomic presence were detected at the order, family, genus, or species levels between *rbcL* and *rbcL*-CF (Table S3); therefore, only a single comparison between microscopy and metabarcoding was presented for alpha diversity.

With microscopy, 116 taxa (with 24% not identified at the species level; five forms of *Achnanthidium* were included in the *Achnanthidium minutissimum* s.l. group; Figure S2) were found, belonging to 9 orders, 24 families, and 37 genera. With metabarcoding, over 500 ASVs were found that were assigned to 10 orders, 20 families, and 28 genera.

With microscopy, 29.2% of taxa occurred only once in all samples while with metabarcoding over 70% of ASVs (*rbcL*: 71.5%; *rbcL*-CF: 80.2) occurred only once in all samples. Less than 1% of ASVs and less than 5% of $taxa_{MIC}$ occurred in all samples (Table S4). By month, many $taxa_{MIC}$ (July: 39%, October: 41%) occurred only in one sample in July and/or October. Similarly, most ASVs (July and October: *rbcL* and *rbcL*-CF: 77%) occurred only in one sample during July and/or October. However, a slightly higher percentage of ASVs and $taxa_{MIC}$ occurred in all seven October samples (*rbcL* and *rbcL*-CF: 2%; $taxa_{MIC}$: 11%) than in all seven July ones (*rbcL* and *rbcL*-CF: 1%; $taxa_{MIC}$: 8%; Table S5).

3.3. Comparison of Alpha Diversity of Microscopy and Metabarcoding Irrespective of Month

At the order level, 9 orders were found with both microscopy and metabarcoding, while one order was only found with metabarcoding (Table 2). At the family level, 19 families were found both with both microscopy and metabarcoding, while one family was only found with metabarcoding and four families only with microscopy (Table 2). In total, 42 genera were found; 23 of them were found with both microscopy and metabarcoding while 5 genera were only found with metabarcoding, and 14 genera only with microscopy (Table 2). At the species level, 15 species were found with both methods, 74 species were unique to microscopy, and 39 species were unique to metabarcoding (Table S6).

Table 2. Overview of orders, families, and genera that were (i) detected by both metabarcoding and microscopy (white background), (ii) unique to metabarcoding (bold and dark grey background), and (iii) unique to microscopy (grey background); family names were abbreviated at the ceae character string (c.); the superscript s indicates taxa occurring only during July, and the superscript a indicates taxa occurring only during October.

Order (n = 10)	**Family (n = 24)**	**Genus (n = 42)**			
Achnanthales	Achnanthidiac.	*Achnanthidium*	*Psammothidium*	***Rossithidium***	*Planothidium*
Aulacoseirales	Aulacoseirac.	*Aulacoseira*			
Bacillariales	Bacillariac.	*Nitzschia*	***Hantzschia***	***Denticula***	
Naviculales	Amphipleurac.	*Frustulia*			
	Brachysirac.	*Brachysira*			
	Naviculac.	*Navicula*	*Caloneis*	*Genkalia*	*Microcostatus*
	Naviculales incertae sedis	*Mayamaea*	*Kobayasiella*	*Microfissurata* [a]	
	Sellaphorac.	*Sellaphora*	*Chamaepinnularia* [s]		
	Pinnulariac.	*Pinnularia*			
	Neidiac.	*Neidium*			
	Diadesmidac.	***Luticola***	*Humidophila*		
	Cavinulac. [s]	*Cavinula* [s]			
	Stauroneidac. [s]	*Stauroneis* [s]			
Cymbellales	Cymbellac.	*Cymbopleura*	*Encyonopsis*		
	Encyonematac.	*Encyonema*			
	Gomphonematac.	*Gomphonema*			
	Witkowskiac.	*Geissleria*			
	Anomoeoneidac. [a]	*Adlafia* [a]			

Table 2. *Cont.*

Order (n = 10)	Family (n = 24)	Genus (n = 42)			
Eunotiales	Eunotiac.	*Eunotia*			
Fragilariales	Fragilariac.	*Fragilaria*	*Odontidium*		
	Staurosirac.	*Staurosira*	*Stauroforma*	*Stauro sirella*	*Pseudostaurosira*
Rhabdonematales	Tabellariac.	*Tabellaria*	*Meridion*		
Surirellales	Surirellac.	*Surirella*			
Thalassiophysales [1]	**Catenulac.** [s]	***Amphora*** [s]			

When comparing microscopy and metabarcoding, 23% of taxa were identified at the genus level using microscopy compared to 22% using metabarcoding. Combining the taxa detection results of both methods (Table 2), the proportion of shared taxa decreased progressively from the order to the species level (order: 90%; family: 79%; genus: 55%; species: 12%).

Of the 42 genera, several genera (n = 22) such as *Achnanthidium*, *Fragilaria*, or *Nitzschia* (Table S7) showed more ASVs per genus with metabarcoding than $taxa_{MIC}$ per genus with microscopy. For these genera, the correction factor (CF)—which reflects the gene copy number—fell into the lower quartile of CF values (Table S3), indicating that the higher ASV diversity was not a methodological sequencing artefact. Only genera unique to microscopy (n = 14) and *Caloneis* (Table S5), which was found with both methods, showed more $taxa_{MIC}$ per genus than ASVs per genus. An equal number (n = 14, 1, 1, respectively) of ASVs per genus as $taxa_{MIC}$ per genus was found for *Psammothidium*, *Mayamaea*, and *Meridion*, respectively (Table S5). Considering the genera with the highest diversity found with microscopy (Table S6), many species found with microscopy (n = 74) were not found with metabarcoding, mostly because of missing reference sequences in Diat.barcode (Table 3).

Table 3. Coverage of species for the top six most diverse genera by microscopy (MIC) and metabarcoding (BAR); genera are ordered by decreasing species richness according to microscopy.

Genus	Detected in MIC	Present in Reference Database	Detected in MIC and BAR	Missing in BAR Reference Database but Detected in MIC
Eunotia	13	13	2	11
Psammothiudium	12	7	1	9
Encynonema	10	13	3	7
Pinnularia	7	48	2	5
Nitzschia	6	70	1	3
Sellaphora	3	17	0	2

3.4. Diatom Red List

Of the 128 taxa identified at the species level by microscopy and metabarcoding, 53 were included in different categories of the diatom Red List. In particular, six species unique to microscopy (*Brachysira calcicola* Lange-Bertalot; *Eunotia praerupta* Ehrenberg 1843; *Neidium alpinum* Hustedt 1943; *Psammothidium levanderi* (Hustedt) Bukhtiyarova & Round 1996; *Psammothidium rechtense* (Leclercq) Lange-Bertalot 1999; *Psammothidium*

scoticum (R.J.Flower & V.J.Jones) Bukhtiyarova & Round 1996) and two species unique to metabarcoding (*Brachysira vitrea* (Grunow) R.Ross 1986; *Navicula angusta* Grunow 1860) were classified as "endangered". Moreover, 11 species were classified as "vulnerable" (one common to microscopy and metabarcoding, nine unique to microscopy and one unique to metabarcoding), 22 species were classified as "threatened to an unknown extent" (one common to microscopy and metabarcoding, 15 unique to microscopy and six unique to metabarcoding) and 12 species were classified as "close to threatened" (four common to microscopy and metabarcoding, six unique to microscopy and two unique to metabarcoding) (Tables 4 and S6).

Table 4. Number of Red List diatom species [53] detected by microscopy (MIC), metabarcoding (BAR), or both.

Status	Common to MIC and BAR	Unique to MIC	Unique to BAR
endangered	0	6	2
vulnerable	1	9	1
threatened to an unknown extent	1	15	6
close to threatened	4	6	2
data deficiency	1	10	5
least concerned	7	13	18

3.5. Biological Quality Assessment

Based on the SPI, good water quality was indicated with metabarcoding and high quality with microscopy for all samples during both months ($p > 0.001$; Table S8). The SPI was not significantly different between July and October for either metabarcoding or microscopy (non-parametric Wilcoxon signed-rank test, $p > 0.05$; Table S8).

3.6. Monthly Differences in Alpha Diversity Assessed with Microscopy and Metabarcoding

The total number of $taxa_{MIC}$ and ASVs was higher in July (ASVs = 417; $taxa_{MIC}$ = 102) than October (ASVs = 252; $taxa_{MIC}$ = 72). Similarly, the mean number of $taxa_{MIC}$ ($p < 0.01$) and ASVs ($p < 0.05$) was higher in July ($taxa_{MIC}$: 27 ± 3; ASVs: 93 ± 37) than in October ($taxa_{MIC}$: 37 ± 7; ASVs: 54 ± 21).

Of the ten orders, Thalassiophysales and its corresponding family and genus were exclusively found in July metabarcoding samples and showed a mean relative abundance <0.1% (Figure 1; Table 2).

The order Surirellales showed a similar low mean relative abundance (<0.25%) with microscopy and metabarcoding and was not found in October microscopy samples (Figure 1). As assessed by two-way ANOVA, Bacillariales and Cymbellales did not show any monthly or methodological differences. Achnanthales, Aulacoseirales, Eunotiales, and Rhabdonematales showed higher mean relative abundance in July than in October, while Fragilariales showed higher values in October than in July with microscopy and metabarcoding; all these orders did not show differences in mean relative abundance between microscopy and metabarcoding (Figure 1; Table 5). For Naviculales, a higher mean relative abundance was found with microscopy than with metabarcoding and a higher mean relative abundance during July than October was only found when comparing *rbcL* to microscopy. For Aulacoseirales, a higher mean relative abundance was found during July than October with microscopy and metabarcoding and a higher mean relative abundance was found with *rbcL* than with microscopy for both months but not with *rbcL*-CF compared to microscopy. Thus, the application of the CF adjusted the mean % values of metabarcoding to values of microscopy only for a low abundance order (% mean abundance < 1%; Aulacoseirales) while for other orders no differences were found.

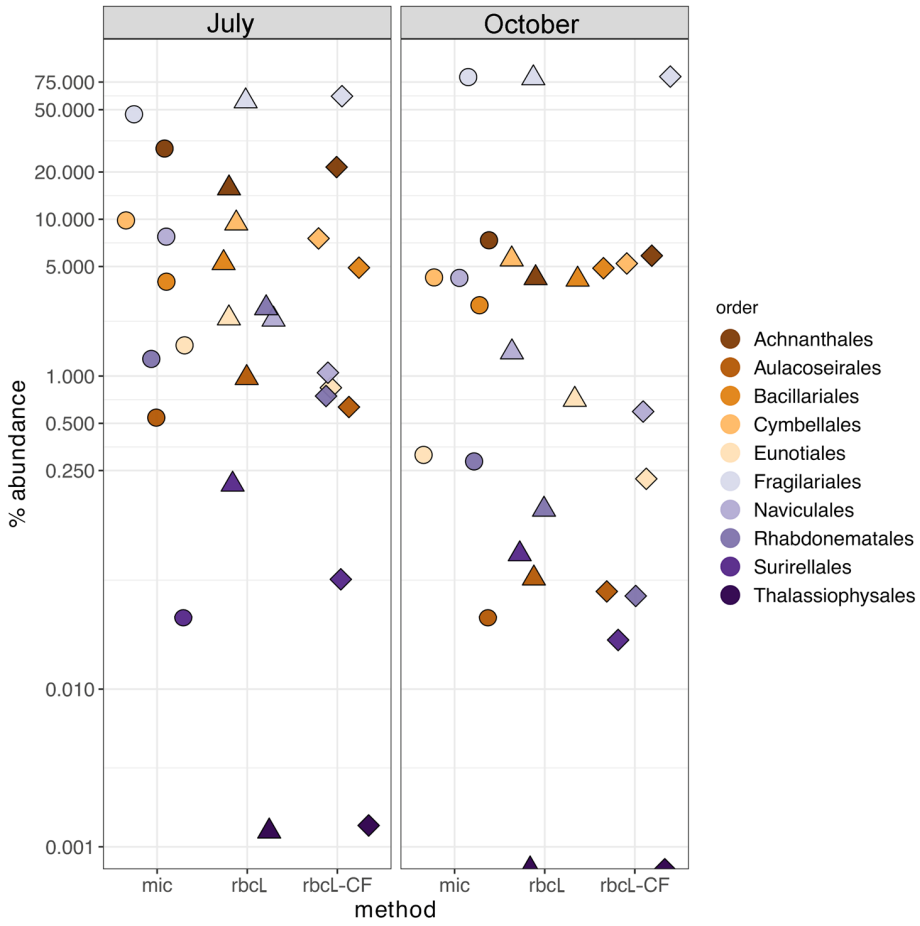


Figure 1. Mean relative abundance of diatom orders detected by microscopy (mic) and metabarcoding across samples, grouped by month and method. Please note that relative abundances below 1% are subject to different detection limits, as microscopy resolution is bound by the total valve count (500 cells per sample), whereas metabarcoding detects low-frequency ASVs at much deeper coverage); circles are for microscopy, triangles for *rbcL*, and diamonds for *rbcL*-CF.

We acknowledge that because of high spatial variability across sampling sites, the relative abundance exhibited considerable variance (Tables 5–8); detailed site-by-site data for each rbcL dataset are provided in Table S9 to allow a direct comparison of local diatom variation.

Of the 24 families found, 14 showed a mean relative abundance <1% in both months with microscopy and metabarcoding. Of these rare families with a mean relative abundance <1%, Cavinulaceae and Stauroneidaceae were exclusively found in July microscopy samples and Anomoeoneidaceae was exclusively found in October microscopy samples (Table 2; Figure 2). Similarly, the family Catenulaceae was exclusively found in July metabarcoding samples and Naviculales incertae sedis was exclusively found in October metabarcoding samples, both with a mean relative abundance <1%. Among low-abundance families, Amphipleuraceae, Catenulaceae, Naviculales incertae sedis, Neidiaceae, and Surirellaceae displayed a mean relative abundance of <0.1% across both microscopic and metabarcoding assessments (Figure 2). While Diadesmidiaceae showed a mean abundance <0.1% with metabarcoding, it had a ten times higher mean relative abundance with microscopy (Figure 2). As assessed by two-way ANOVA, Achnanthidiaceae, Aulacoseiraceae, Eunotiaceae, Gomphonemataceae, and Tabellariaceae showed a higher mean relative abundance in July than in October while Fragilariaceae showed a higher mean relative abundance in October than in July with metabarcoding and microscopy (Figure 2; Table 6). Cymbellaceae showed a higher October mean relative abundance than July values only with

rbcL (interaction between month and method; $p < 0.05$) compared to microscopy while microscopy and *rbcL*-CF did not show a seasonal difference. Similarly, Pinnulariaceae showed higher July than October values only with microscopy compared to *rbcL*-CF (interaction between month and method; $p < 0.05$) while microscopy and *rbcL* did not show a month effect. Brachysiraceae, Diadesmidaceae, and Naviculaceae showed a lower mean relative abundance with metabarcoding than microscopy while Cymbellaceae showed a higher mean relative abundance with metabarcoding than with microscopy. Pinnulariaceae and Sellaphoraceae showed a lower mean relative abundance with *rbcL*-CF than microscopy but not between *rbcL* and microscopy, while Aulacoseiraceae and Staurosiraceae showed a higher mean relative abundance with *rbcL* than with microscopy but not between *rbcL*-CF and microscopy (Table 6). Thus, for the high-abundance families (Fragilariaceae to Tabellariaceae; Table 6) the application of the CF had an influence on monthly differences only once (Staurosiraceae) while for low-abundance families (mean abundance around 1%) more differences were found. The mean relative abundance of unassigned orders was not different ($p > 0.05$) between July and October (Table 6).

Table 5. Relative abundance of orders grouped by month and method (mean ± one standard deviation across all samples). The orders are ordered from highest to lowest relative abundance. Orders Thalassiophysales and Surellales are excluded (mean relative abundance < 0.1%) and the former occurred only in October. A non-parametric two-way ANOVA (month and method effect) was used to test for differences between *rbcL* and microscopy (mic) and between *rbcL*-CF and microscopy; thus, the column "month" indicates a month effect between *rbcL* and microscopy (MIC) ($month_{rbcL\ vs.\ MIC}$) and between *rbcL*-CF and microscopy ($month_{rbcL\text{-}CF\ vs.\ MIC}$), respectively. The column effect (eff.) indicates if a monthly difference (S) and/or a methodological difference (M) was found, and ° indicates that the difference was not found in both comparisons. Different uppercase letters (A, B) as superscripts indicate a statistically significant monthly difference for both methods. Different lowercase letters (a, b) as superscripts in the columns *rbcL* and *rbcL*-CF, respectively, indicate a statistically significant method effect. Significance levels are $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*), where no letters indicate no effect ($p > 0.05$). NA indicates no taxonomic assignment at the order level. Oct is October.

Eff.	Order	$Month_{rbcL\ vs.\ MIC}$	*rbcL*	MIC	*rbcL*-CF	$Month_{rbcL\text{-}CF\ vs.\ MIC}$
S	Fragilariales	July ^{A***} Oct ^{B}	57 ± 13.8 79 ± 9.5	47 ± 17.8 81 ± 9	61 ± 17.8 81 ± 10.2	July ^{A***} Oct ^{B}
S	Achnanthales	July ^{A***} Oct ^{B}	16 ± 13.8 4 ± 6.1	28 ± 16.7 7 ± 4.9	22 ± 17.7 6 ± 8.4	July ^{A***} Oct ^{B}
	Cymbellales	July Oct	9 ± 11 6 ± 5.2	10 ± 10 4 ± 5.3	8 ± 9.6 5 ± 5.4	July Oct
	Bacillariales	July Oct	5 ± 5 4 ± 3.3	4 ± 3.2 3 ± 1.6	5 ± 4.6 5 ± 4.3	July Oct
S	Rhabdonematales	July ^{A***} Oct ^{B}	3 ± 4.3 0 ± 0.2	1 ± 1.9 0 ± 0.4	1 ± 1.2 0 ± 0.1	July ^{A*} Oct ^{B}
S	Eunotiales	July ^{A**} Oct ^{B}	2 ± 1.8 1 ± 0.8	2 ± 1.4 0 ± 0.4	1 ± 0.7 0 ± 0.2	July ^{A**} Oct ^{B}
S °M	Naviculales	July ^{A*} Oct ^{B}	2 ± 1.6 ^{a***} 1 ± 0.5 ^{a***}	8 ± 6.4 ^{b} 4 ± 2.3 ^{b}	1 ± 0.9 ^{a***} 1 ± 0.3 ^{a***}	July Oct
SM °	Aulacoseirales	July ^{A***} Oct ^{B}	0.9 ± 1.4 ^{a*} 0.05 ± 0.136 ^{a*}	0.5 ± 0.7 ^{b} 0.02 ± 0.076 ^{b}	0.6 ± 0.83 0.04 ± 0.11	July ^{A*} Oct ^{B}
	NA	July Oct	4 ± 4 4 ± 4		2 ± 1.6 2 ± 1.9	July Oct

Table 6. Mean relative abundance of families grouped by month and method (mean ± one standard deviation across all samples). The families Amphipleuraceae, Catenulaceae, Naviculales *incertae sedis*, Neidiaceae, and Surirellaceae are excluded because they show a mean relative abundance <0.1%. Families are ordered from highest to lowest relative abundance. A non-parametric two-way ANOVA (month and method effect) was used to test for differences between *rbcL* and microscopy (MIC) ($month_{rbcL\ vs.\ MIC}$) and between *rbcL*-CF and MIC ($month_{rbcL\text{-}CF\ vs.\ MIC}$). The column effect (eff) indicates if a monthly difference (S) and/or a methodological difference (M) was found, and ° indicates that the difference was not found in both comparisons. Uppercase letters A and B as superscripts indicate a statistically significant monthly difference for both methods, while letters C and D indicate a statistically significant monthly difference only for one method (i.e., an interaction effect). Different lowercase letters (a, b) as superscripts in the columns *rbcL* and *rbcL*-CF, respectively, indicate a statistically significant method effect. Significance levels are $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*), where no letters indicate no effect ($p > 0.05$). NA indicates no taxonomic assignment at the order level. Oct is October.

Eff.	Family	$Month_{rbcL\ vs.\ MIC}$	*rbcL*	MIC	*rbcL*-CF	$Month_{rbcL\text{-}CF\ vs.\ MIC}$
S	Fragilariac.	July ^{A***} Oct ^{B}	40 ± 10.7 53 ± 13.4	37 ± 17.8 69 ± 7.4	50 ± 14.8 63 ± 11.8	July ^{A***} Oct ^{B}
S °M °	Staurosirac.	July ^{A*} Oct ^{B}	16 ± 7 ^{a***} 26 ± 11 ^{a***}	9 ± 3.3 ^{b} 12 ± 3.9 ^{b}	11 ± 5.6 18 ± 7.9	July Oct
S	Achnanthidic.	July ^{A***} Oct ^{B}	16 ± 13.8 4 ± 6.1	28 ±16.7 7 ± 4.9	22 ± 17.6 6 ± 8.4	July ^{A***} Oct ^{B}
	Bacillariac.	July Oct	5 ± 5 4 ± 3.3	4 ± 3.2 3 ± 1.6	5 ± 4.6 5 ± 4.3	July Oct
	Encyonematac.	July Oct	5 ± 10.2 4 ± 4.7	7 ± 10.2 4 ± 5.2	5 ± 9.5 4 ± 4.8	July Oct
S	Gomphonematac.	July ^{A***} Oct ^{B}	4 ± 4.2 1 ± 0.9	3 ± 5 0 ± 0.3	2 ± 3 0 ± 0.5	July ^{A*} Oct ^{B}
S	Eunotiac.	July ^{A**} Oct ^{B}	2 ± 1.8 1 ± 0.8	2 ± 1.4 0 ± 0.4	1 ± 0.7 0 ± 0.2	July ^{A**} Oct ^{B}
S	Tabellariac.	July ^{A***} Oct ^{B}	3 ± 4.3 0 ± 0.2	1 ± 1.9 0 ± 0.4	1 ± 1.2 0 ± 0.1	July ^{A*} Oct ^{B}
SM °	Aulacoseirac.	July ^{A**} Oct ^{B}	1 ± 1.4 ^{a*} 0 ± 0.1 ^{a*}	1 ± 0.7 ^{b} 0 ± 0.1 ^{b}	1 ± 0.8 0 ± 0.1	July ^{A*} Oct ^{B}
S °M	Cymbellac.	July ^{C*} Oct ^{D}	0 ± 0.2 ^{a*} 1 ± 0.6 ^{a*}	0 ± 0.2 ^{b} 0 ± 0.2 ^{b}	0 ± 0.4 ^{a**} 1 ± 0.8 ^{a**}	July Oct
S °M °	Pinnulariac.	July Oct	1 ± 0.5 0 ± 0.5	1 ± 0.8 ^{b} 0 ± 0.3 ^{b}	0 ± 0.2 ^{a***} 0 ± 0.1 ^{a***}	July ^{C***} Oct ^{D}
M	Naviculac.	July Oct	0 ± 0.2 ^{a**} 0 ± 0.02 ^{a**}	1 ± 1.2 ^{b} 1 ± 0.4 ^{b}	0 ± 0.1 ^{a***} 0 ± 0.005 ^{a***}	July Oct
M	Brachysirac.	July Oct	0 ± 0.4 ^{a**} 0 ± 0.02 ^{a**}	1 ± 0.9 ^{b} 1 ± 0.6 ^{b}	0 ± 0.3 ^{a**} 0 ± 0.02 ^{a**}	July Oct
M °	Sellaphorac.	July Oct	0 ± 0.3 0 ± 0.4	1 ± 0.8 ^{b} 1 ± 0.3 ^{b}	0 ± 0.2 ^{a*} 0 ± 0.3 ^{a*}	July Oct
M	Diadesmidac.	July Oct	0.03 ± 0.05 ^{a***} 0.02 ± 0.03 ^{a***}	3 ± 5.7 ^{b} 2 ± 2.3 ^{b}	0.03 ± 0.05 ^{a***} 0.02 ± 0.03 ^{a***}	July Oct
	NA	July Oct	5 ± 3.8 4 ± 3.9		2 ± 1.6 2 ± 1.9	July Oct

Table 7. Relative abundance of the top seven most abundant genera found with microscopy (MIC) and metabarcoding across samples, grouped by month and method (mean ± one standard deviation over all samples). Genera are ordered from highest to lowest mean relative abundance. A non-parametric two-way ANOVA (month and method effect) was used to test for differences between *rbcL* and mic and between *rbcL*-CF and mic; thus, the column month indicates monthly difference between *rbcL* and microscopy ($month_{rbcL\ vs.\ mic}$) and between *rbcL*-CF and microscopy ($month_{rbcL\text{-}CF\ vs.\ mic}$), respectively. Column effect (eff) indicates if a monthly difference (S) and/or a methodological difference (M) was found, and ° indicates that the difference was not found in both comparisons. Uppercase letters A and B as superscripts on months indicate a statistically significant monthly difference for both compared methods, while letters C and D as superscripts on the column mic indicate a statistically significant monthly difference only for microscopy (i.e., interaction effect). For the method effect, different lowercase letters (a, b) in the columns *rbcL* and *rbcL*-CF, respectively, indicate a statistically significant methodological difference. Significance levels are $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*), where no letters indicate no effect ($p > 0.05$). NA indicates no taxonomic assignment at the order level. Oct is October.

Eff.	Genera	$Month_{rbcL\ vs.\ MIC}$	*rbcL*	MIC	*rbcL*-CF	$Month_{rbcL\text{-}CF\ vs.\ MIC}$
S	*Fragilaria*	July ^{A***} Oct ^{B}	40 ± 10.5 50 ± 12.4	37 ± 17.8 69 ± 7.6	50 ± 14.7 62 ± 11.5	July ^{A***} Oct ^{B}
SM	*Staurosira*	July Oct	16 ± 7 $^{a*** C**}$ 26 ± 11 $^{a*** D}$	3 ± 1.7 ^{b} 4 ± 3.3 ^{b}	11 ± 5.6 ^{a***} 18 ± 7.9 ^{a***}	July ^{A**} Oct ^{B}
	Encyonema	July Oct	6 ± 10.1 5 ± 5	7 ± 10.2 4 ± 5.2	5 ± 9.4 5 ± 5.1	July Oct
	Nitzschia	July Oct	5 ± 5 4 ± 3.3	4 ± 3.2 3 ± 1.6	5 ± 4.6 5 ± 4.3	July Oct
S	*Gomphonema*	July ^{A***} Oct ^{B}	4 ± 4.2 1 ± 0.9	3 ± 5 0 ± 0.3	2 ± 3 0 ± 0.5	July ^{A**} Oct ^{B}
SM °	*Achnanthidium*	July Oct	2 ± 1.5 ^{a*} 1 ± 1.1	6 ± 5.7 $^{b C*}$ 1 ± 0.8 ^{D}	3 ± 2.3 1 ± 1.4	July ^{A**} Oct ^{B}
SM	*Psammothidium*	July Oct	2 ± 1.6 ^{a**} 0 ± 0.5 ^{a**}	22 ± 18.6 $^{b C*}$ 6 ± 4.9 $^{b D}$	2 ± 2.3 ^{a**} 1 ± 0.8 ^{a**}	July ^{A*} Oct ^{B}
	NA	July Oct	17 ± 13.5 8 ± 6.5		18 ± 15.4 6 ± 7	July Oct

Table 8. Relative abundance (mean ± one standard deviation over all samples) of the top seven species assessed with microscopy and metabarcoding grouped by month. Species are ordered from highest to lowest mean relative abundance. Month testing was done with the non-parametric Wilcoxon signed-rank test; significance levels are $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*), and no stars indicate no effect ($p > 0.05$).

Method	Species	July	October	Month Diff.
rbcL	*Staurosira venter*	12.5 ± 8.21	21.6 ± 11.29	**
	Encyonema minutum	5.0 ± 9.46	4.3 ± 4.67	
	Nitzschia acidoclinata	3.2 ± 3.48	3.4 ± 2.99	
	Gomphonema minutum	2.8 ± 4.41	0.1 ± 0.27	
	Psammothidium chlidanos	1.6 ± 1.57	0.5 ± 0.52	
	Achnanthidium minutissimum s.l.	1.4 ± 0.94	0.5 ± 1.04	
	Odontidium mesodon	0.2 ± 0.27	3.6 ± 9.49	

Table 8. *Cont.*

Method	Species	July	October	Month Diff.
microscopy	*Fragilaria* cf. *nanana*	36.9 ± 17.55	68.7 ± 7.80	***
	Psammothidium subatomoides	10.2 ± 11.61	2.8 ± 3.18	
	Achnanthidium minutissimum	6.2 ± 5.73	1.1 ± 0.83	*
	P. microscopicum	4.1 ± 4.20	0.7 ± 0.66	
	Encyonema brevicapitatum	3.7 ± 6.28	1.6 ± 2.48	
	Humidophila schmassmannii	3.2 ± 5.71	1.9 ± 2.30	
	Nitzschia perminuta	3.2 ± 2.68	2.2 ± 1.16	

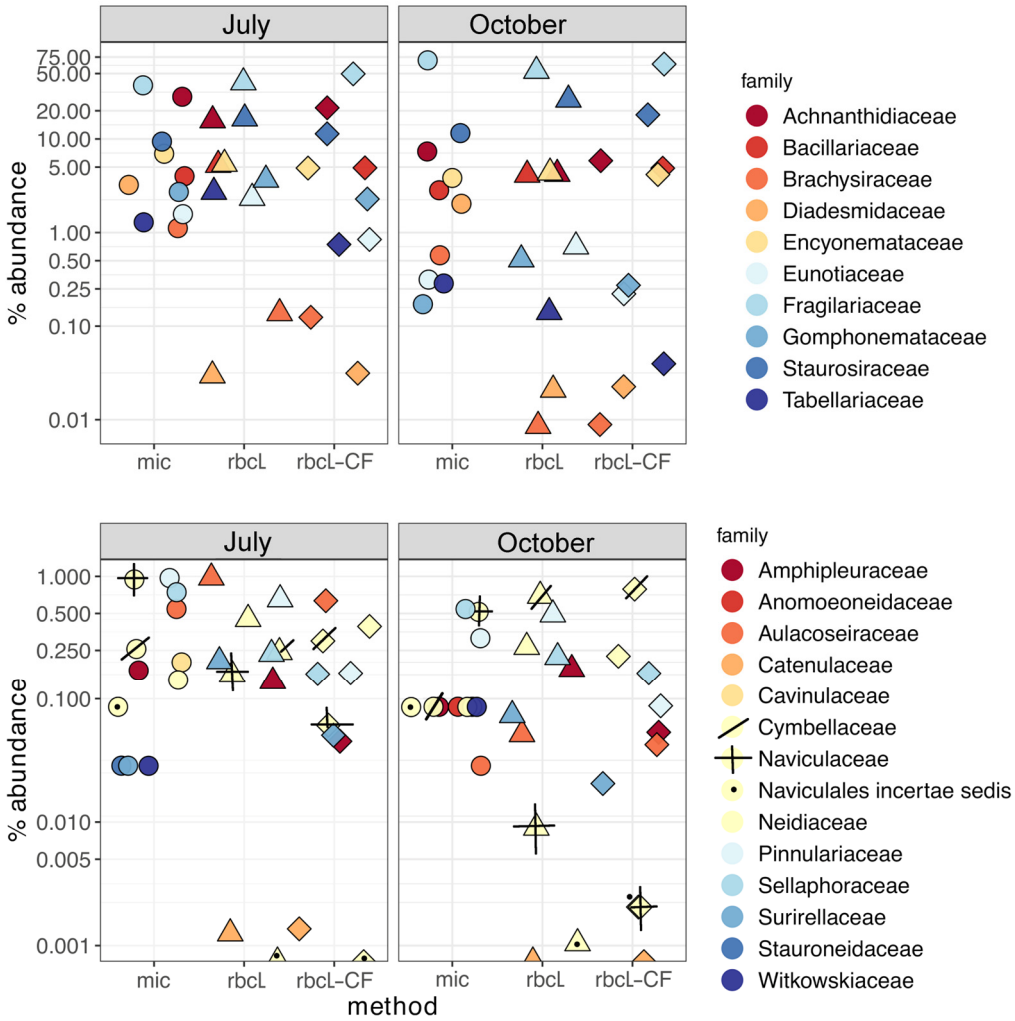


Figure 2. Mean relative abundance of diatom families detected via microscopy (mic) and metabarcoding across samples, grouped by month and method. Upper panel: top 10 most abundant families; lower panel: families with mean relative abundance <1%. Please note that relative abundances below 1% are subject to different detection limits, as microscopy resolution is bound by the total valve count (500 cells per sample), whereas metabarcoding detects low-frequency ASVs at much deeper coverage; circles are for microscopy, triangles for *rbcL*, and diamonds for *rbcL*-CF.

Out of the 42 genera, 23 were detected in both July and October using both microscopy and metabarcoding. Three genera (*Cavinula*, *Stauroneis*, *Chamaepinnularia*) were exclusively found in July and two genera (*Adlafia* and *Microfissurata*) in October by microscopy while one genus (*Amphora*) was only found in July by metabarcoding (Table 2). In total, 29 genera showed a mean relative abundance <1% in both months (Figure 3). However, while

Brachysira generally showed a mean relative abundance <1%, it exceeded 1% in July when using microscopy (Figure 3).

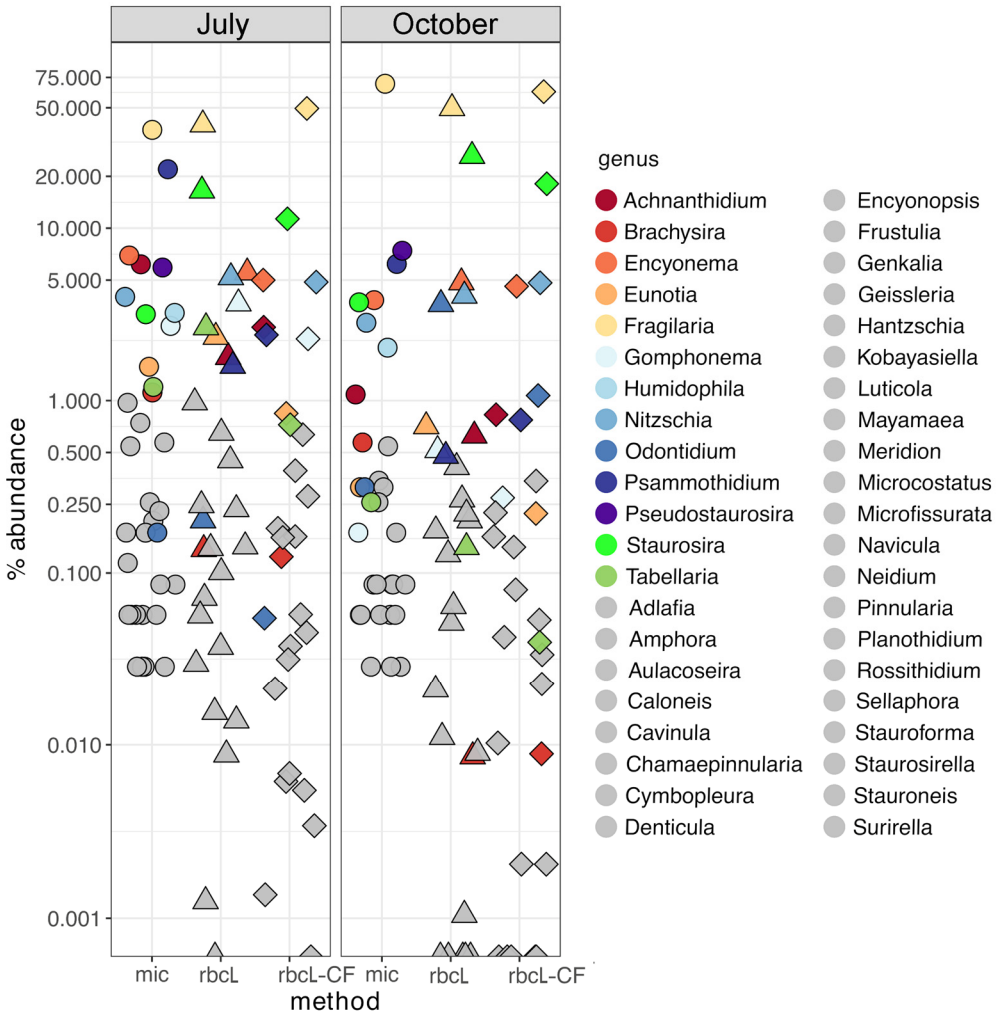


Figure 3. Mean relative abundance of diatom genera detected by microscopy (mic) and metabarcoding across samples, grouped by month and method. Grey colour indicates genera with a mean relative abundance <1% in all samples and coloured symbols indicate genera with a mean relative abundance >1% in either July or October samples. Please note that relative abundances below 1% are subject to differential detection limits, as microscopy resolution is bound by the total valve count (500 cells per sample), whereas metabarcoding detects low-frequency ASVs at much deeper coverage); circles are for microscopy, triangles for *rbcL*, and diamonds for *rbcL*-CF.

Among the seven genera with the highest mean relative abundance found by microscopy and metabarcoding, *Encyonema* and *Nitzschia* did not show any monthly or methodological differences (two-way ANOVA, $p > 0.05$), and *Gomphonema* showed higher values during July than in October with both methods (Figure 3; Table 7). The mean relative abundance of unassigned genera by metabarcoding was not different ($p > 0.05$) between July and October (Table 7).

When comparing *rbcL* to microscopy, *Staurosira* showed higher mean relative abundance with *rbcL* than with microscopy, both in July and October; however, October showed a higher mean relative abundance than July only with *rbcL* (interaction effect, $p < 0.05$). When comparing *rbcL*-CF to microscopy, *Staurosira* showed higher mean relative abundance with *rbcL* than microscopy and October showed higher mean relative abundance than July. The larger variability of *rbcL*-CF was responsible for the significant month effect (Table 7).

Psammothidium showed a lower mean relative abundance with *rbcL* than with microscopy, in both months, when comparing *rbcL* to microscopy; however, July showed a higher mean relative abundance than October only with microscopy (interaction effect, $p < 0.05$) when comparing *rbcL* to microscopy and *rbcL*–CF to microscopy, respectively.

When comparing *rbcL* to microscopy, *Achnanthidium* showed a higher mean relative abundance with microscopy than with *rbcL*, and July showed a higher mean relative abundance than October only with microscopy. When comparing *rbcL*-CF to microscopy, *Achnanthidium* showed a higher mean relative abundance in July than in October but no differences between methods.

Thus, focusing on the top 7 genera, the application of the CF did not change methodological differences in four case; in two cases values remained smaller, and in one case values became larger than with microscopy.

A comparison between species abundance assessed with microscopy and metabarcoding was difficult because of the high percentage (47%) of unassigned ASVs at the species level. Among the taxonomically assigned species, most $taxa_{MIC}$ had a mean relative abundance smaller than 1% and the top seveb taxa from microscopy showed a mean abundance between 0.6 ± 0.79% (*Psammothidium microscopicum* (Cholnoky) S.Blanco 2016 during October) and 69 ± 7.8% (*Fragilaria* cf. *nanana* Lange-Bertalot 1993 during October; Table 8). Similarly, most ASVs with species assignment had a low relative abundance, and also the top seven species both with *rbcL* and *rbcL*-CF showed a mean relative abundance between 0.2 ± 0.27% (*rbcL*-CF: *Odontidium mesodon* (Ehrenberg) Kützing 1849 during July) and 22 ± 11.2% (*rbcL*: *Staurosira venter* (Ehrenberg) Cleve & J.D.Möller 1879 during October; Table 8).

The mean relative abundance of the top seven species did not differ significantly between *rbcL* and *rbcL*-CF (Table S9). Among the $species_{metabarcoding}$, only *Gomphonema minutum* (C.Agardh) C.Agardh 1831 showed a higher mean relative abundance during July than October (Table 7). Among the $species_{MIC}$, only *A. minutissimum* s.l. showed higher mean relative abundance during July, and *F.* cf. *nanana* showed a higher mean relative abundance during October (Table 8).

The mean relative abundance of *Encyonema minutum* D.G.Mann 1990 (metabarcoding) showed a similar relative abundance as *Encyonema brevicapitatum* Krammer 1997 (microscopy), while *A. minutissimum* s.l. had a lower relative abundance with metabarcoding than with microscopy, especially during July (Figure 3; Table 8). The mean relative abundance of *Fragilaria* from metabarcoding (Table 7) comprising 68 ASVs was similar to the mean relative abundance of *F.* cf. *nanana* from microscopy (Tables 7 and 8) because other $species_{MIC}$ of *Fragilaria* (*F. radians*, *F. campyla*, *Fragilaria* sp.) had a mean July and/or October relative abundance <0.25%.

3.7. Ecological Guilds

Using both microscopy and metabarcoding, diatoms of the high-profile guild dominated in July and October (mean relative abundance > 50%). Based on microscopy, the mean relative abundance of the high-profile guild was higher in October than in July, whereas diatoms of the motile guild showed higher relative abundance in July than in October (Table 9). In contrast, ecological guilds showed no monthly differences when using metabarcoding (Table 9).

Table 9. Mean relative abundance (mean ± one standard deviation over all samples) of ecological guilds (high profile—H; low profile—L; motile—M) grouped by month and method. Monthly testing was done with the non-parametric Wilcoxon signed-rank sum test; significance levels are $p < 0.01$ (**), $p < 0.05$ (*), and no letters indicate no effect ($p > 0.05$); different letters indicated statistically significant differences. With metabarcoding not all ASVs could be attributed to guilds (NA).

Ecological Guild	Microscopy		*rbcL*		*rbcL*-CF	
	July	October	July	October	July	October
H	60 ± 1.5 ^{a**}	85 ± 7.2 ^{b}	72 ± 18.7	85 ± 10.2	70 ± 21.3	86 ± 10.6
L	28 ± 16.6	7 ± 4.8	4 ± 2.6	1 ± 1.3	6 ± 3.7	2 ± 1.7
M	11.6 ± 6.7 ^{a*}	7 ± 3.5 ^{b}	7 ± 4.3	5 ± 3.8	6 ± 4.2	5 ± 4.5
NA			17 ± 13.5	8 ± 6.5	18 ± 15	6 ± 6.9

3.8. Indicator Species for Months

Using microscopy, only a single indicator species for July (*Aulacoseira alpigena* (Grunow) Krammer 1991; A = 1.0; B = 0.86; $p < 0.01$) was identified, which was present in all July samples and absent in October. No $taxon_{MIC}$ was an indicator for October samples.

With *rbcL*, five ASVs were found as indicators for July (unique to metabarcoding: ASV 20 = *Aulacoseira distans* (Ehrenberg) Simonsen 1979; ASV 36 = *Nitzschia* sp.; found with microscopy and metabacoring: ASV 53 = *A. alpigena*; ASV 67 = Bacillariophyta; ASV 89 = *Pinnularia microstauron* (Ehrenberg) Clevel 1891; log_2Fold change > 10; $p < 0.01$). While all were absent in October, the first three occurred in four, five, and four July samples, respectively, and the others in only three July samples. With *rbcL*-CF, seven ASVs were found as indicators for July (log_2Fold change > 10; $p < 0.001$). The first three July indicators were the same as for *rbcL*, and the others (found with microscopy and metabarcoding: ASV 73—*S. venter*; ASV 77—*S. venter*; unique to metabarcoding: ASV 101—*Rossithidium anastasiae* (Kaczmarska) Potapova 2012; ASV 109—*Fragilaria* sp.) occurred in only two July samples.

Using *rbcL*, only one ASV (ASV 40 = Bacillariophyta; log_2Fold change = −12; $p < 0.001$) was found as an indicator for October. ASV 40 was present in all October samples and absent in July. Using rbcL-CF, only one ASV (ASV 169 = *Fragilaria* sp.; log_2Fold change = −22; $p < 0.001$) was found as an indicator for October, which occurred in only two October samples and was absent in July.

3.9. NMDS

NMDS showed a clear separation of months both with metabarcoding and microscopy (Figure 4). With environmental fitting, ordinations with metabarcoding and microscopy were related to temperature ($NMDS_{MIC}$: $R^2 = 0.69$; $p < 0.01$; $NMDS_{rbcL}$: $R^2 = 0.69$; $p < 0.01$; $NMDS_{rbcL\text{-}CF}$: $R^2 = 0.59$; $p < 0.05$), pH ($NMDS_{MIC}$: $R^2 = 0.69$; $p < 0.01$; $NMDS_{rbcL}$: $R^2 = 0.45$; $p < 0.05$), and conductivity ($NMDS_{MIC}$: $R^2 = 0.66$; $p < 0.01$; $NMDS_{rbcL}$: $R^2 = 0.42$; $p < 0.05$). High values of temperature and pH were linked to July and high values of conductivity to October samples. In Procrustes analysis, NMDS ordinations of *rbcL* and *rbcL*-CF data were correlated (r = 0.96; $p < 0.001$) while NMDS ordinations of microscopy and metabarcoding were not correlated (r < 0.35; $p > 0.05$).

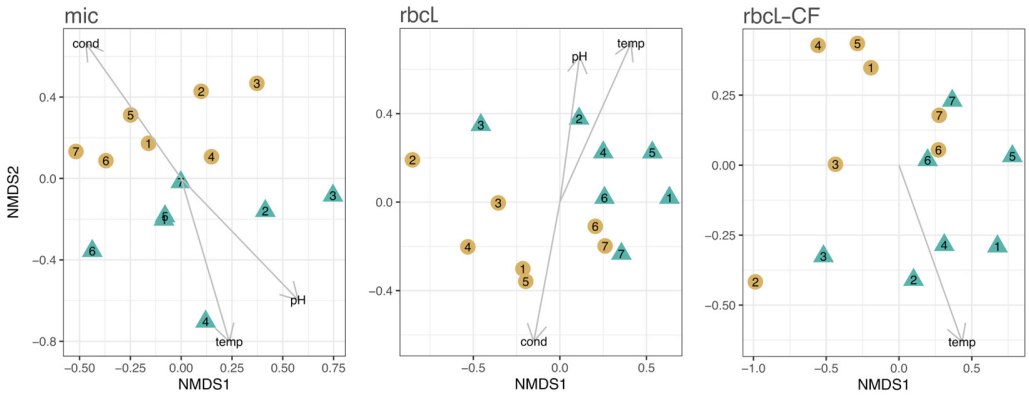


Figure 4. NMDS ordination with microscopy (mic; stress = 0.18) and metabarcoding ($stress_{rbcL}$ = 0.13; $stress_{rbcL\text{-}CF}$ = 0.12). Several environmental variables (water temperature—temp; conductivity—cond) were related to the ordinations. Triangles are July samples and circles are October samples; numbers indicate sampling site identity.

4. Discussion

4.1. Characterization of Upper Lake Balma

Little is known about the concordance between microscopy and metabarcoding for assessing benthic diatom diversity in mountain lakes of the Italian Alps. Here, we compared both methods in Upper Lake Balma in July, representative of summer, and October, representative of autumn. This lake, despite being a remote lake, is affected by cattle grazing, fish introduction [36–38], and microplastic pollution [37]. The observed high phosphorus concentrations in Upper Lake Balma were typical of mesotrophic conditions and might be linked to cattle and fish activity. As expected, water temperature dropped by nearly 50% and dissolved oxygen saturation by 11% whereas conductivity increased by 27% from July to October. Increased conductivity in mountain lakes can be attributed to summer evaporation [71], and decreased water temperature to water column cooling. The observed oxygen decrease was negligible considering its small change, and values were in line with those observed in other alpine lakes of the Western Alps [72]. By sampling 63 mountain lakes from siliceous or calcareous geology in the French Alps, distinct lake categories were established based on diatom composition [73]. Upper Lake Balma, characterized by its siliceous metamorphic ophiolites geology and its close proximity to the French Alps [73], fits into the category of shallow Alpine lakes (sensu [73]), but the reported dominant and indicator diatoms do not fully align with our findings. In fact, genera such as *Fragilaria* and *Staurosira* were less abundant in French lakes compared to Upper Lake Balma, further corroborating the existing knowledge gap for benthic diatoms of mountain lakes in the Italian Alps and a huge spatial variability.

4.2. Alpha Diversity of Diatoms

We observed a higher diversity with metabarcoding than with microscopy, although metabarcoding missed certain taxa detected by microscopy, as highlighted in previous studies (e.g., [25,27,74,75]). Nevertheless, a similar percentage of taxa were identified at the genus level by microscopy and metabarcoding as found by [23,24] while metabarcoding had almost twice as many unidentified taxa at species level as microscopy. Here, genera found by microscopy but not by metabarcoding (e.g., *Planothidium*, *Microcostatus*) were present in the dedicated diatom barcode reference library, while some genera found with microscopy were missing (i.e., *Genkalia*, *Kobayasiella*, *Cavinula*) or were underrepresented in the reference library (e.g., *Psammothidium*, typical of a circumneutral, oligotrophic, siliceous substrate environment [76]; *Eunotia*, an indicator of ecological integrity and naturalistic value [77]). The non-detection of genera present in the reference library likely stems from

molecular biases, such as PCR primer mismatches, differential DNA extraction efficiency, or low target template abundance relative to dominant taxa. Taxa of Diadesmidiaceae were hardly detected by metabarcoding, attributable to their heavily silicified frustules resisting standard DNA extraction lysis, alongside potential PCR primer mismatches or gaps in reference databases for aerophilic and high-altitude species. Similarly, the taxonomic identity of the most abundant diatom species in Upper Lake Balma differed between the metabarcoding and microscopic assessments, as observed for Fennoscandinavian lakes and rivers because traditional morphotaxa are split into several ASVs with distinct ecological profiles [16]. One reason for this is the so-called concealed diversity (i.e., morphologically identical taxa can show considerable genetic diversity). The discussion of whether this genetic variation indicates real cryptic species or rather genetic diversity in populations is ongoing [78]. We also observed that the number of ASVs per genus was substantially higher than the number of morphotaxa ($taxa_{MIC}$) per genus, confirming that metabarcoding detects greater biodiversity than microscopy. The genus *Fragilaria* dominated both with metabarcoding and microscopy. However, with microscopy, the highest abundance was found for *F.* cf. *nanana*, most ASVs (62 out of 68 ASVs) of *Fragilaria* were not assigned to the species level and only a few sequences were assigned to *F. nanoides* Lange-Bertalot 1996 (n = 2), *F. gracilis* Østrup 1910 (n = 2), and *F. bidens* Heiberg 1863 (n = 1). This suggests a high level of undescribed *Fragilaria* diversity in mountain lakes like Upper Lake Balma. In summary, our results point to several limitations in metabarcoding, such as primer bias, sequence divergence between Alpine species and deposited reference sequences, and a lack of reference data requiring further species culturing and sequencing, issues frequently reported in metabarcoding studies (e.g., [27,28,74]). Similarly, the five morphological forms of *Achnanthidium* included in the *A. minutissimum* s.l. group likely require formal taxonomic description. Indeed, high genetic diversity within *Achnanthidium* is well documented [18].

Combining the diatom diversity assessed by metabarcoding and microscopy in Lake Balma, 53 out of 128 species were identified as worthy of protection (*sensu* [49]), highlighting the lake's high ecological value. When using the SPI to evaluate water quality, microscopy yielded a 'high' ecological status, whereas metabarcoding indicated a 'good' status. Such discrepancies have frequently been reported in previous studies [26–28] and can be attributed to the lower species-level identification success of metabarcoding.

4.3. Ecological Guilds

The relatively low species-level assignment rate of ASVs also affected the ecological guild assessment of diatoms. Specifically, microscopy revealed shifts in guild composition (i.e., motile guild diatoms increased in July, while the high-profile guild increased in October despite remaining dominant from July to October), whereas metabarcoding detected no monthly differences. Nevertheless, both methods consistently showed a dominance of the high-profile guild in both July and October. In general, diatoms belonging to the high-profile guild are adapted to turbulent environments and high nutrient concentrations [58], while motile diatoms are adapted to high nutrient concentrations and low current velocities [3]. In alpine lakes, however, diatoms of the high-profile guild and unattached and motile diatoms are predominantly found in lakes with low turbulence and long water residence times [79]. We suggest that Upper Lake Balma was generally a turbulent environment during July and October 2021, as its shallow depth might have favoured repeated mixing by summer storms alongside convective mixing driven by water cooling in autumn. Furthermore, the water column likely exhibited a relatively greater stability in autumn than in summer. We acknowledge that without direct meteorological and physical water-column data, this hypothesis remains speculative; nevertheless, the observed shifts in guild composition strongly pointed to changes in stability. We ruled out

nutrient availability as a driver of guild change, as no monthly differences in nutrient levels were detected.

4.4. Monthly Differences in Diatom Diversity

Diatom taxon richness was higher in July than in Otober. Likewise, most families, orders, and genera reached higher relative abundances in July, with *Fragilaria* and *Staurosira* being the abundant genera dominating in October (two-way ANOVA). Thus, diatom communities in Lake Balma exhibited monthly differences similar to those observed in other mountain lakes, which are often linked to water temperature (e.g., [80,81]). Furthermore, *Fragilaria* is common in sediments of Alpine lakes, *F. gracilis* and *F. nanoides* are linked to low water temperature and mixing [82], and *Fragilaria* is a diatom of the high-profile guild. Thus, the increase of the high-profile guild in October can be related to the increase of *Fragilaria*.

In the indicator species analysis, which evaluates samples individually, unlike a two-way ANOVA relying on sample variance, few morphotaxa ($taxa_{MIC}$) and ASVs were identified as monthly indicators. Nonetheless, a higher number of ASVs than $taxa_{MIC}$ served as monthly indicators, which we suggest was related to the higher overall diversity detected by metabarcoding. The only indicator species identified by microscopy was *Aulacoseira alpigena*, which was likewise detected as a July indicator via metabarcoding. This species is traditionally considered characteristic of acidic, oligotrophic environments [83,84], matching only marginally with the overall mesotrophic classification of Upper Lake Balma. This apparent discrepancy may be explained by localized littoral microhabitats, or by a broader ecological tolerance of *A. alpigena* in high-altitude lake ecosystems in response to stressful conditions [84]. Several ASVs were July indicator taxa while only one was identified for October, in accordance with a higher diversity during July than October. Among July ASV indicators in Lake Balma, *Staurosira venter* is linked to high summer water temperatures [82], supporting its role as an indicator for July conditions. The only October ASV indicator identified by metabarcoding belonged to *Fragilaria*, further supporting the genus's preference for October.

4.5. Utility of Using the CF for Metabarcoding

We applied CFs to assess the usefulness of the method to balance primer bias for a dataset with incomplete taxonomic resolution across various levels, as is often the case in metabarcoding studies. We acknowledge that using a CF from another taxonomic level than species can lead to misleading results because the CF for species of the same genus, family, or higher level can vary a lot. Nevertheless, we applied this method to assess its usefulness considering a dataset with an incomplete taxonomic assignment at all taxonomic levels. At the order level, the mean relative abundance did not differ significantly between microscopy and metabarcoding, with two exceptions: Naviculales, for which both *rbcL* and *rbcL*-CF showed a lower relative abundance than microscopy, and Aulacoseriales, where *rbcL*-CF was more similar to the estimates of microscopy. For most families with high relative abundance, applying the CF made no significant difference, except for Staurosiraceae, where *rbcL*-CF yielded results similar to microscopy. Similarly, for rare families, applying the CF offered no clear advantage. For the most abundant genera, no clear advantage of the CF method was found. For the water quality assessment, the application of the CF did not influence the metabarcoding-based SPI, which was generally lower than that based on microscopy. Therefore, applying the CF did not significantly improve the agreement between metabarcoding and microscopy data in Upper Lake Balma. Our findings contrasted with previous studies (e.g., [26] and references therein), underscoring the distinct ecological characteristics of mountain lakes.

4.6. Month and Site Differences in Multivariate Ordination

When using a taxonomy-independent approach such as NMDS, both metabarcoding and microscopy revealed monthly differences linked to similar environmental variables (pH, temperature, conductivity). Benthic diatoms in alpine lakes are known to be strongly associated with pH [82,85], water temperature drives diatom succession [33], and pH alongside conductivity determines diatom community composition [82,86]. Thus, the influence of these variables on the diatoms of Lake Balma was not surprising. However, the multivariate ordination of samples in NMDS differed between metabarcoding and microscopy, indicating distinct biodiversity assessments at the individual sample level.

5. Conclusions

The combined use of microscopy and metabarcoding provided a higher diversity than using a single method, emphasizing the unexplored high diversity of alpine lakes. Furthermore, metabarcoding provided a higher diversity compared to microscopy, but not all taxa found with microscopy were detected via metabarcoding and vice versa. For metabarcoding, the application of the CF based on diatom biovolume did not provide a clear advantage with respect to microscopy. Nevertheless, both methods consistently reflected monthly patterns and environmental influences. Thus, an integrated approach provides a more comprehensive assessment of diatom diversity as already pointed out [23], although it requires greater financial and personnel resources. While many studies highlight the limitations of metabarcoding reference databases [22,26,27,75,87], filling these databases depends on taxonomists providing verified sequence data. We suggest that, alongside advancing alternative metabarcoding methodologies [21], halting the ongoing loss of taxonomic expertise [29] is crucial for effectively assessing diatom diversity in lake ecosystems, particularly in mountain environments.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ecologies7040111/s1, Figure S1: Upper panel: general geographic location; lower panel: bathymetric map of Upper Lake Balma with the location of the sampling sites; Figure S2: Light micrographs *Achnanthidium minutissimum* s.l. (1–5). *Achnanthidium minutissimum* s.l. formeA. (6–10). *Achnanthidium minutissimum* s.l. formeB (11–14). *Achnanthidium minutissimum* s.l. formeC. (15–19). *Achnanthidium minutissimum* s.l. formedD. (20–24). Scale bar is 10 µm; Table S1: Primers used; Table S2: Site specific environmental variables; % dissolved oxygen saturation (% DO sat), dissolved oxygen (DO), conductivity (cond; µS cm^{-1}); total P, NH_4, and NO_3 in mgL^{-1}; Table S3: ASV tables; Table S4: % occurrence of ASVs and $taxa_{MIC}$ in samples; Table S5: % occurrence of ASVs and taxa in samples split by months; Table S6: List of taxa identified at species level common and unique to microscopy (MIC) and metabarcoding (BAR) and their attribution according to the diatom red list: endangered = EN; vulnerable = VU; threatened to an unknown extent = T; close to threatened = CT; data deficiency = DD; least concerned = LC; Table S7: Number of ASVs per genus and number of morphologically identified taxa ($taxa_{MIC}$) per genus; Table S8: Mean SPI for months and assessed with microscopy and metabarcoding. Different lowercase letters in the column *rbcL* and *rbcL*-CF, respectively, indicate a statistically significant method effect. Significant levels are $p < 0.001$ (***); Table S9: rbcL (OTU_tables_rbcL.xlsx) and rbcL-CF (OTU_tables_rbcL.CF.xlsx).

Author Contributions: Conceptualization, U.O. and F.C.; methodology, F.C., C.C., E.P., M.P. (Massimo Pindo), S.B., M.B., P.P. and M.P. (Massimo Prearo); software, U.O.; validation, F.C., C.C., E.P., M.P. (Massimo Pindo), S.B., M.B., P.P. and M.P. (Massimo Prearo); formal analysis, U.O. and F.C.; investigation, F.C., C.C., E.P., M.P. (Massimo Pindo), S.B., M.B., P.P. and M.P. (Massimo Prearo); resources, F.C. and C.C.; data curation, F.C., C.C., E.P., M.P. (Massimo Pindo), S.B., M.B., P.P. and M.P. (Massimo Prearo); funding acquisition, F.C. and C.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the institutional research budget of ECOAMB (Fondazione Edmund Mach). This research received no external funding.

Data Availability Statement: Environmental data and code are available from the corresponding author upon request. The sequence data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB91740.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Rivera-Rondón, C.A.; Catalan, J. Diatoms as Indicators of the Multivariate Environment of Mountain Lakes. *Sci. Total Environ.* **2020**, *703*, 135517. [CrossRef] [PubMed]
2. Rimet, F.; Bouchez, A.; Tapolczai, K. Spatial Heterogeneity of Littoral Benthic Diatoms in a Large Lake: Monitoring Implications. *Hydrobiologia* **2016**, *771*, 179–193. [CrossRef]
3. Rimet, F.; Bouchez, A. Life-Forms, Cell-Sizes and Ecological Guilds of Diatoms in European Rivers. *Knowl. Manag. Aquat. Ecosyst.* **2012**, *406*, 1. [CrossRef]
4. Moser, K.A.; Baron, J.S.; Brahney, J.; Oleksy, I.A.; Saros, J.E.; Hundey, E.J.; Sadro, S.A.; Kopáček, J.; Sommaruga, R.; Kainz, M.J.; et al. Mountain Lakes: Eyes on Global Environmental Change. *Glob. Planet. Change* **2019**, *178*, 77–95. [CrossRef]
5. Agnarsson, I.; Kuntner, M. Taxonomy in a Changing World: Seeking Solutions for a Science in Crisis. *Syst. Biol.* **2007**, *56*, 531–539. [CrossRef] [PubMed]
6. Catalan, J.; Camarero, L.; Felip, M.; Pla, S.; Ventura, M.; Buchaca, T.; Bartumeus, F.; Mendoza, G.D.; Miró, A.; Casamayor, E.O.; et al. High Mountain Lakes: Extreme Habitats and Witnesses of Environmental Changes. *Limnetica* **2006**, *25*, 551–584. [CrossRef]
7. Tolotti, M. Phytoplankton and Littoral Epilithic Diatoms in High Mountain Lakes of the Adamello-Brenta Regional Park (Trentino, Italy) and Their Relation to Trophic Status and Acidification Risk. *J. Limnol.* **2001**, *60*, 171–188. [CrossRef]
8. Ciutti, F.; Blanco, S.; Bertoli, M.; Pizzul, E.; Pastorino, P.; Prearo, M.; Cappelletti, C. Diatom diversity in a mountain lake in southern European Alps (Italy). *Phytotaxa* **2025**, *710*, 1–21. [CrossRef]
9. Lange-Bertalot, H.; Metzeltin, D. Indicators of Oligotrophy-800 Taxa Representative of Three Ecologically Distinct Lake Types, Carbonate Buffered-Oligodystrophic-Weakly Buffered Soft Water. In *Iconographia Diatomologica. Annotated Diatom Micrographs*; Lange-Bertalot, H., Ed.; Koeltz Scientific Books: Königstein, Germany, 1996; Volume 2.
10. Rivera-Rondón, C.A.R.; Catalan, J. Diatom Diversity in the Lakes of the Pyrenees: An Iconographic Reference. *Limnetica* **2017**, *36*, 127–395. [CrossRef]
11. Blanco, S.; Pla-Rabès, S.; Wetzel, C.E.; Granados, I. A New *Psammothidium* Species (Bacillariophyta, Achnanthidiaceae) from Cimera Lake (Gredos Mountain Range), Central Spain. *Cryptogam. Algol.* **2017**, *38*, 17–29. [CrossRef]
12. Wetzel, C.E.; Ector, L. Two New *Punctastriata* (Bacillariophyta) Species from Subalpine French Lakes. *Bot. Lett.* **2020**, *168*, 42–55. [CrossRef]
13. Peszek, L.; Robinson, C.T.; Rybak, M.; Kawecka, B. *Orthoseira helvetica* sp. nov.—A new diatom (Bacillariophyceae) from the Swiss Alps. *Diatom Res.* **2023**, *38*, 77–87. [CrossRef]
14. Solak, C.N.; Hamilton, P.B.; Peszek, Ł.; Yilmaz, E.; Ertorun, N.; Blanco, S. A new diatom (Bacillariophyta) species—*Sellaphora lucectoriana* sp. nov.—From a glacial lake in Kaçkar Mountain (Eastern Blacksea Region), Rize, Republic of Türkiye. *Nova Hedwig.* **2023**, *117*, 203–212. [CrossRef]
15. Vassal, V.; Heudre, D.; Wetzel, C.E.; Tudesque, L. Two New *Achnanthidium* (Bacillariophyceae) Species from a Recent Alpine Lake in the National Park of Ecrins (South of France). *Nova Hedwig.* **2023**, *117*, 143–163. [CrossRef]
16. Kahlert, M.; Bailet, B.; Chonova, T.; Karjalainen, S.M.; Schneider, S.C.; Tapolczai, K. Same Same, but Different: The Response of Diatoms to Environmental Gradients in Fennoscandian Streams and Lakes–Barcodes, Traits and Microscope Data Compared. *Ecol. Indic.* **2021**, *130*, 108088. [CrossRef]
17. Trobajo, R.; Rovira, L.; Ector, L.; Wetzel, C.E.; Kelly, M.; Mann, D.G. Morphology and Identity of Some Ecologically Important Small *Nitzschia* Species. *Diatom Res.* **2013**, *28*, 37–59. [CrossRef]
18. Rimet, F.; Pinseel, E.; Bouchez, A.; Japoshvili, B.; Mumladze, L. Diatom Endemism and Taxonomic Turnover: Assessment in High-Altitude Alpine Lakes Covering a Large Geographical Range. *Sci. Total Environ.* **2023**, *871*, 161970. [CrossRef] [PubMed]
19. Rivera, S.F.; Vasselon, V.; Jacquet, S.; Bouchez, A.; Ariztegui, D.; Rimet, F. Metabarcoding of Lake Benthic Diatoms: From Structure Assemblages to Ecological Assessment. *Hydrobiologia* **2018**, *807*, 37–51. [CrossRef]
20. Apothéloz-Perret-Gentil, L.; Cordonier, A.; Straub, F.; Iseli, J.; Esling, P.; Pawlowski, J. Taxonomy-free molecular diatom index for high-throughput eDNA biomonitoring. *Mol. Ecol. Resour.* **2017**, *17*, 1231–1242. [CrossRef] [PubMed]
21. Tapolczai, K.; Rimet, F.; Ćirić, M.; Ballot, A.; Laplace-Treyture, C.; Alric, B. A Novel Framework for Phytoplankton Biomonitoring: Trait Assignment of 23S rRNA Sequences. *Ecol. Indic.* **2025**, *173*, 113361. [CrossRef]

22. Kaleli, A.; Ozbayram, G.; Akcaalan, R. Comparative Investigation of Marine Benthic Diatom Assemblages with Morphological and Metabarcoding Approaches Along the Coasts of Istanbul Metropolis. *Estuaries Coasts* **2023**, *47*, 473–484. [CrossRef]
23. Wang, B.; Li, R.; Lan, X.; Kong, D.; Liu, X.; Xie, S. Benthic Diatom eDNA Metabarcoding for Ecological Assessment of an Urban River: A Comparison with Morphological Method. *Ecol. Indic.* **2024**, *166*, 112302. [CrossRef]
24. Pérez-Burillo, J.; Valoti, G.; Witkowski, A.; Prado, P.; Mann, D.G.; Trobajo, R. Assessment of Marine Benthic Diatom Communities: Insights from a Combined Morphological–Metabarcoding Approach in Mediterranean Shallow Coastal Waters. *Mar. Pollut. Bull.* **2022**, *174*, 113183. [CrossRef] [PubMed]
25. Rimet, F.; Vasselon, V.; Keszte, B.A.; Bouchez, A. Do We Similarly Assess Diversity with Microscopy and High-Throughput Sequencing? Case of Microalgae in Lakes. *Org. Divers. Evol.* **2018**, *18*, 51–62. [CrossRef]
26. Duleba, M.; Földi, A.; Micsinai, A.; Várbíró, G.; Mohr, A.; Sipos, R.; Szabó, G.; Buczkó, K.; Trábert, Z.; Kiss, K.T.; et al. Applicability of Diatom Metabarcoding in the Ecological Status Assessment of Hungarian Lotic and Soda Pan Habitats. *Ecol. Indic.* **2021**, *130*, 108105. [CrossRef]
27. Bailet, B.; Bouchez, A.; Franc, A.; Frigerio, J.-M.; Keck, F.; Karjalainen, S.-M.; Rimet, F.; Schneider, S.; Kahlert, M. Molecular versus Morphological Data for Benthic Diatoms Biomonitoring in Northern Europe Freshwater and Consequences for Ecological Status. *MBMG* **2019**, *3*, e34002. [CrossRef]
28. Kamberović, J.; Gligora Udovič, M.; Kulaš, A.; Tapolczai, K.; Orlić, S.; Jusufović, A.; Gajić, A.; Žutinić, P.; Ahmić, A.; Kalamujić Stroil, B. The Diatom Diversity and Ecological Status of a Tufa-Depositing River through eDNA Metabarcoding vs. a Morphological Approach—A Case Study of the Una River (Bosnia and Herzegovina). *Microorganisms* **2024**, *12*, 1722. [CrossRef] [PubMed]
29. Bertoli, M.; Pizzul, E.; Basile, S.; Perilli, S.; Tauler, R.; Lacorte, S.; Prearo, M.; Pastorino, P. Littoral Macrobenthic Invertebrates of Two High-Altitude Lakes in the Alps: A Small-Scale Analysis. *Ecohydrol. Hydrobiol.* **2023**, *23*, 211–223. [CrossRef]
30. Salvi, G.; Bertoli, M.; Giubileo, C.; Pastorino, P.; Pavoni, E.; Crosera, M.; Prearo, M.; Pizzul, E. Testate Amoeba and Chironomid Assemblages from Balma Lake (Piedmont, Italy): A Multi-Proxy Record to Identifying Recent Climate and Environmental Changes in Alpine Areas. *Quat. Sci. Rev.* **2022**, *285*, 107547. [CrossRef]
31. Pastorino, P.; Anselmi, S.; Esposito, G.; Bertoli, M.; Pizzul, E.; Barceló, D.; Elia, A.C.; Dondo, A.; Prearo, M.; Renzi, M. Microplastics in Biotic and Abiotic Compartments of High-Mountain Lakes from Alps. *Ecol. Indic.* **2023**, *150*, 110215. [CrossRef]
32. Löbl, I.; Klausnitzer, B.; Hartmann, M.; Krell, F.-T. The Silent Extinction of Species and Taxonomists—An Appeal to Science Policymakers and Legislators. *Diversity* **2023**, *15*, 1053. [CrossRef]
33. Vasselon, V.; Bouchez, A.; Rimet, F.; Jacquet, S.; Trobajo, R.; Corniquel, M.; Tapolczai, K.; Domaizon, I. Avoiding Quantification Bias in Metabarcoding: Application of a Cell Biovolume Correction Factor in Diatom Molecular Biomonitoring. *Methods Ecol. Evol.* **2018**, *9*, 1060–1069. [CrossRef]
34. Rimet, F.; Gusev, E.; Kahlert, M.; Kelly, M.G.; Kulikovskiy, M.; Maltsev, Y.; Mann, D.G.; Pfannkuchen, M.; Trobajo, R.; Vasselon, V.; et al. Diat.barcode, an Open-Access Curated Barcode Library for Diatoms. *Sci. Rep.* **2019**, *9*, 15116. [CrossRef] [PubMed]
35. Cantonati, M.; Hofmann, G.; Spitale, D.; Werum, M.; Lange-Bertalot, H. Diatom Red Lists: Important Tools to Assess and Preserve Biodiversity and Habitats in the Face of Direct Impacts and Environmental Change. *Biodivers. Conserv.* **2022**, *31*, 453–477. [CrossRef]
36. Balma, G.A.C.; Delmastro, G.B.; Forneris, G. Segnalazione di alcune specie ittiche esotiche d'importazione in Italia settentrionale, con particolare riferimento alle acque piemontesi (Pisces: Osteichthyes). *Atti Soc. Ital. Sci. Nat. Mus. Civ. Stor. Nat. Milano* **1989**, *130*, 109–116.
37. Pastorino, P.; Prearo, M.; Bertoli, M.; Menconi, V.; Esposito, G.; Righetti, M.; Mugetti, D.; Pederiva, S.; Abete, M.C.; Pizzul, E. Assessment of Biological and Sanitary Condition of Alien Fish from a High-Mountain Lake (Cottian Alps). *Water* **2020**, *12*, 559. [CrossRef]
38. Perilli, S.; Pastorino, P.; Bertoli, M.; Salvi, G.; Franz, F.; Prearo, M.; Pizzul, E. Correction to: Changes in Midge Assemblages (Diptera Chironomidae) in an Alpine Lake from the Italian Western Alps: The Role and Importance of Fish Introduction. *Hydrobiologia* **2020**, *847*, 2977. [CrossRef]
39. Wang, X.; Liu, Y.; Zhang, Y.; Wu, P.; Liu, X.; Nan, F.; Liu, Q.; Lv, J.; Feng, J.; Xie, S. The Complex Relationships between Diatoms, Bacterial Communities, and Dissolved Organic Matter: Effects of Silicon Concentration. *Algal Res.* **2024**, *79*, 103460. [CrossRef]
40. Guiry, M.D.; Guiry, G.M. AlgaeBase. *Choice Rev. Online* **2025**, *42*, 42sup-0247.
41. Chardon, C.; Vasselon, V.; Vautier, M.; Lacroix, S.; Bouchez, A.; Rimet, F.; Domaizon, I. Diatom DNA Library Preparation for Illumina Miseq Sequencing Using the rbcL Marker Gene. 2020. Available online: https://www.protocols.io/view/diatom-dna-library-preparation-for-illumina-miseq-kqdg3573zv25/v1 (accessed on 30 June 2025).
42. Vasselon, V.; Rimet, F.; Tapolczai, K.; Bouchez, A. Assessing Ecological Status with Diatoms DNA Metabarcoding: Scaling-up on a WFD Monitoring Network (Mayotte Island, France). *Ecol. Indic.* **2017**, *82*, 1–12. [CrossRef]
43. Martin, M. Cutadapt Removes Adapter Sequences from High-Throughput Sequencing Reads. *EMBnet. J.* **2011**, *17*, 10–12. [CrossRef]

44. Tapolczai, K.; Selmeczy, G.B.; Szabó, B.; B-Béres, V.; Keck, F.; Bouchez, A.; Rimet, F.; Padisák, J. The Potential of Exact Sequence Variants (ESVs) to Interpret and Assess the Impact of Agricultural Pressure on Stream Diatom Assemblages Revealed by DNA Metabarcoding. *Ecol. Indic.* **2021**, *122*, 107322. [CrossRef]
45. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-Resolution Sample Inference from Illumina Amplicon Data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef] [PubMed]
46. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
47. Keck, F. *diatbarcode: Access the diat.barcode Database*, version 0.0.0.9000; GitHub: San Francisco, CA, USA, 2020.
48. Nielsen, K.M.; Johnsen, P.J.; Bensasson, D.; Daffonchio, D. Release and Persistence of Extracellular DNA in the Environment. *Environ. Biosaf. Res.* **2007**, *6*, 37–53. [CrossRef]
49. Hofmann, G.; Lange-Bertalot, H.; Werum, M.; Klee, R. Rote Liste der limnischen Kieselalgen. *Naturschutz Biol. Vielfalt* **2018**, *70*, 601–708.
50. Cemagref. Étude des méthodes biologiques quantitative d'appréciation de la qualité des eaux. In *Rapport Division Qualité des Eaux Lyon*; Agence financière de Bassin Rhône-Méditerranée-Corse: Lyon, France, 1982; p. 218.
51. Tison-Rosebery, J.; Boutry, S.; Bertrin, V.; Leboucher, T.; Morin, S. A New Diatom-based Multimetric Index to Assess Lake Ecological Status. *Environ. Monit. Assess.* **2023**, *195*, 1202. [CrossRef] [PubMed]
52. Ács, É.; Földi, A.; Vad, C.F.; Trábert, Z.; Kiss, K.T.; Duleba, M.; Borics, G.; Grigorszky, I.; Botta-Dukát, Z. Trait-Based Community Assembly of Epiphytic Diatoms in Saline Astatic Ponds: A Test of the Stress-Dominance Hypothesis. *Sci. Rep.* **2019**, *9*, 15749. [CrossRef] [PubMed]
53. Kelly, M.; Acs, E.; Bertrin, V.; Bennion, H.; Borics, G.; Burgess, A.; Denys, L.; Ecke, F.; Kahlert, M.; Karjalainen, S.; et al. *Water Framework Directive Intercalibration Technical Report: Lake Phytobenthos Ecological Assessment Methods*; Publications Office of the European Union: Luxembourg, 2014; p. 125.
54. Ector, L.; Rimet, F. Using Bioindicators to Assess Rivers in Europe: An Overview. In *Modelling Community Structure in Freshwater Ecosystems*; Springer: Berlin/Heidelberg, Germany, 2005; Volume 1, pp. 7–19. [CrossRef] [PubMed]
55. Feio, M.J.; Almeida, S.F.; Craveiro, S.C.; Calado, A.J. A Comparison between Biotic Indices and Predictive Models in Stream Water Quality Assessment Based on Benthic Diatom Communities. *Ecol. Indic.* **2009**, *9*, 497–507. [CrossRef]
56. Blanco, S. What Do Diatom Indices Indicate? Modeling the Specific Pollution Sensitivity Index. *Environ. Sci. Pollut. Res.* **2024**, *31*, 29449–29459. [CrossRef] [PubMed]
57. Gelis, N.M.; Sathicq, M. *Diathor: Calculate Ecological Information and Diatom Based Indices*, version 0.1.5; Comprehensive R Archive Network (CRAN): Vienna, Austria, 2024.
58. Passy, S.I. Diatom Ecological Guilds Display Distinct and Predictable Behavior along Nutrient and Disturbance Gradients in Running Waters. *Aquat. Bot.* **2007**, *86*, 171–178. [CrossRef]
59. McGill, B.J.; Enquist, B.J.; Weiher, E.; Westoby, M. Rebuilding Community Ecology from Functional Traits. *Trends Ecol. Evol.* **2006**, *21*, 178–185. [CrossRef] [PubMed]
60. Weiss, S.; Xu, Z.Z.; Peddada, S.; Amir, A.; Bittinger, K.; Gonzalez, A.; Lozupone, C.; Zaneveld, J.R.; Vázquez-Baeza, Y.; Birmingham, A.; et al. Normalization and Microbial Differential Abundance Strategies Depend upon Data Characteristics. *Microbiome* **2017**, *5*, 27. [CrossRef] [PubMed]
61. Yan, L. *ggvenn: Draw Venn Diagram by "ggplot2"*, version 0.1.19; Comprehensive R Archive Network (CRAN): Vienna, Austria, 2023.
62. Chen, H. *VennDiagram: Generate High-Resolution Venn and Euler Plots*, version 1.8.2; Comprehensive R Archive Network (CRAN): Vienna, Austria, 2022.
63. Kay, M.; Elkin, L.; Higgins, J.; Wobbrock, J. *ARTool: Aligned Rank Transform for Nonparametric Factorial ANOVAs*, version 0.11.0; Comprehensive R Archive Network (CRAN): Vienna, Austria, 2021.
64. Cáceres, M.D.; Legendre, P. Associations between Species and Groups of Sites: Indices and Statistical Inference. *Ecology* **2009**, *90*, 3566–3574. [CrossRef] [PubMed]
65. Love, M.I.; Huber, W.; Anders, S. Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [CrossRef]
66. Schurch, N.J.; Schofield, P.; Gierliński, M.; Cole, C.; Sherstnev, A.; Singh, V.; Wrobel, N.; Gharbi, K.; Simpson, G.G.; Owen-Hughes, T.; et al. How Many Biological Replicates Are Needed in an RNA-Seq Experiment and Which Differential Expression Tool Should You Use? *RNA* **2016**, *22*, 839–851. [CrossRef] [PubMed]
67. Oksanen, J.; Blanchet, F.G.; Kindt, R.; Legendre, P.; Minchin, P.R.; O'hara, R.B.; Simpson, G.L.; Solymos, P.; Stevens, M.H.H.; Wagner, H. *Package 'Vegan'. Community Ecology Package*, version 2.0; Comprehensive R Archive Network (CRAN): Vienna, Austria, 2013.
68. Ma, J. Joint microbial and metabolomic network estimation with the censored gaussian graphical model. *Stat. Biosci.* **2021**, *13*, 351–372. [CrossRef] [PubMed]

69. Gloor, G.B.; Macklaim, J.M.; Pawlowsky-Glahn, V.; Egozcue, J.J. Microbiome Datasets Are Compositional: And This Is Not Optional. *Front. Microbiol.* **2017**, *8*, 2224. [CrossRef] [PubMed]
70. Neuwirth, E. *RColorBrewer: ColorBrewer Palettes*, version 1.1-3; Comprehensive R Archive Network (CRAN): Vienna, Austria, 2022.
71. Schreder, S.; Sommaruga, R.; Psenner, R.; Chimani, B.; Ganekind, M.; Koinig, K.A. Changes in Air Temperature, but Not in Precipitation, Determine Long-Term Trends in Water Chemistry of High Mountain Lakes of the Alps with and without Rock Glacier Influence. *Sci. Total Environ.* **2023**, *905*, 167750. [CrossRef] [PubMed]
72. Tiberti, R.; Tartari, G.; Marchetto, A. Geomorphology and Hydrochemistry of 12 Alpine Lakes in the Gran Paradiso National Park, Italy. *J. Limnol.* **2010**, *69*, 242–256. [CrossRef]
73. Feret, L.; Bouchez, A.; Rimet, F. Benthic Diatom Communities in High Altitude Lakes: A Large Scale Study in the French Alps. *Ann. Limnol.-Int. J. Limnol.* **2017**, *53*, 411–423. [CrossRef]
74. Obertegger, U.; Flaim, G.; Corradini, S.; Cerasino, L.; Zohary, T. Multi-Annual Comparisons of Summer and under-Ice Phytoplankton Communities of a Mountain Lake. *Hydrobiologia* **2022**, *849*, 4613–4635. [CrossRef]
75. Rimet, F.; Abarca, N.; Bouchez, A.; Kusber, W.H.; Jahn, R.; Kahlert, M.; Keck, F.; Kelly, M.G.; Mann, D.G.; Piuz, A.; et al. The Potential of High-Throughput Sequencing (HTS) of Natural Samples as a Source of Primary Taxonomic Information for Reference Libraries of Diatom Barcodes. *Fottea* **2018**, *18*, 37–54. [CrossRef]
76. Cantonati, M.; Zorza, R.; Bertoli, M.; Pastorino, P.; Salvi, G.; Platania, G.; Prearo, M.; Pizzul, E. Recent and Subfossil Diatom Assemblages as Indicators of Environmental Change (Including Fish Introduction) in a High-Mountain Lake. *Ecol. Indic.* **2021**, *125*, 107603. [CrossRef]
77. Cantonati, M.; Lange-Bertalot, H. Diatom Monitors of Close-to-Pristine, Very-Low Alkalinity Habitats: Three New *Eunotia* Species from Springs in Nature Parks of the South-Eastern Alps. *J. Limnol.* **2011**, *70*, 209–221. [CrossRef]
78. Zimmermann, J.; Glöckner, G.; Jahn, R.; Enke, N.; Gemeinholzer, B. Metabarcoding vs. Morphological Identification to Assess Diatom Diversity in Environmental Studies. *Mol. Ecol. Resour.* **2015**, *15*, 526–542. [CrossRef] [PubMed]
79. Rimet, F.; Feret, L.; Bouchez, A.; Dorioz, J.-M.; Dambrine, E. Factors Influencing the Heterogeneity of Benthic Diatom Communities along the Shoreline of Natural Alpine Lakes. *Hydrobiologia* **2019**, *839*, 103–118. [CrossRef]
80. Wang, J.; Li, J.; Cao, Q.; Rioual, P.; Lei, G.; Cai, B.; Zou, Y.; Luo, H.; Li, Q.; Wang, L. Seasonal Variations of Diatoms in a Low-Latitude Mountain Lake: A Case Study from Douhu Lake-Southeast China. *Diatom Res.* **2022**, *37*, 165–178. [CrossRef]
81. Wang, Q.; Yang, X.; Anderson, N.J.; Ji, J. Diatom seasonality and sedimentation in a subtropical Alpine lake (Lugu Hu, Yunnan-Sichuan, Southwest China). *Arct. Antarct. Alp. Res.* **2015**, *47*, 461–472. [CrossRef]
82. Schmidt, R.; Kamenik, C.; Lange-Bertalot, H.; Klee, R. *Fragilaria* and *Staurosira* (Bacillariophyceae) from Sediment Surfaces of 40 Lakes in the Austrian Alps in Relation to Environmental Variables, and Their Potential for Palaeoclimatology. *J. Limnol.* **2004**, *63*, 171–189. [CrossRef]
83. Pedraza Garzon, E.L.; Saros, J.E. Ecology of the Diatom *Aulacoseira pusilla* in Oligotrophic Mountain Lakes, with Implications for Paleoclimate Reconstructions. *Arct. Antarct. Alp. Res.* **2022**, *54*, 147–162. [CrossRef]
84. Fu, W.; Shu, Y.; Yi, Z.; Su, Y.; Pan, Y.; Zhang, F.; Brynjolfsson, S. Diatom Morphology and Adaptation: Current Progress and Potentials for Sustainable Development. *Sustain. Horiz.* **2022**, *2*, 100015. [CrossRef]
85. Lepori, F.; Tolotti, M. Effects of Nitrogen on Benthic Diatom Assemblages in High-Elevation Central and Eastern Alpine Lakes. *Arct. Antarct. Alp. Res.* **2023**, *55*, 2270821. [CrossRef]
86. Soininen, J. Environmental and Spatial Control of Freshwater Diatoms—A Review. *Diatom Res.* **2007**, *22*, 473–490. [CrossRef]
87. Obertegger, U. Temporal and Spatial Differences of the Under-Ice Microbiome Are Linked to Light Transparency and Chlorophyll-a. *Hydrobiologia* **2022**, *849*, 1593–1612. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.