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A novel framework for phytoplankton biomonitoring: Trait assignment of 23S rRNA sequences

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ABSTRACT

Phytoplankton is a key biological group used to assess the ecological status of lakes in several legislative water management plans. Two cutting-edge approaches for community characterization are DNA metabarcoding and trait-based analyses. While the former provides a fast, cost-effective and high-throughput methodology for identifying communities, the latter reveals the structure of communities through bio-ecological traits. The main aim of this study was to combine these approaches to directly assign traits to amplicon sequence variants. To achieve this, we used the newly developed Phytool v3 reference database. Using an *in silico* test, we assessed the efficiency and reliability of our approach. We found: (1) that a greater number of sequences with better reliability can be assigned to traits than to genus or species level and (2) that traits are conserved in the phylogeny with varying extent. Then, we tested the usefulness of direct trait assignment on environmental samples from lakes. The test showed a greater number of successfully assigned sequences and a good ecological interpretation of community structures in the different environments. Furthermore, we identified three factors (completeness of the reference library, sequence similarity and the number of neighbours in the reference database) which, depending on the trait under consideration, interfere with the assignment success of our approach. While DNA metabarcoding data can be exploited in many ways depending on the objectives, our study showed that an innovative framework based on direct trait assignment of sequences could overcome gaps in reference databases and further improve our knowledge of phytoplankton community structure.

1. Introduction

The ecosystem services provided by freshwaters, such as water and food supply, climate regulation, tourism and recreation are essential for human existence and well-being (Grizzetti et al., 2016; Maes et al., 2016; Vári et al., 2022). Due to the unsustainable human activities, freshwater ecosystems are exposed to increased pressures that affect biodiversity (Albert et al., 2021; Pereira et al., 2012). Moreover, biodiversity plays a key role in supporting stability and ecosystem resilience (Amorim & Moura, 2021; Boyer et al., 2009; Oliver et al., 2015). Many countries have therefore implemented special measures to assess the impacts of environmental changes and mitigate their effects on freshwater ecosystems (Kopf et al., 2015) with the objective to maintain ecosystem services (Carvalho et al., 2019). The Water Framework Directive (WFD) has provided a common regulatory framework for the implementation of a water management policy in Europe (European Commission, 2000). The central tenet of the WFD is to implement the strategies needed to achieve and/or maintain the good ecological status of aquatic ecosystems. To meet these expectations, in addition to monitoring physico-chemical and hydromorphological parameters, different organism groups, known as Biological Quality Elements (BQEs), are included in ecological status assessment (Hering et al., 2006).

Phytoplankton is an important biological compartment of the biosphere in terms of biomass (Bar-On et al., 2018). It is highly diverse

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(Borics et al., 2014; Reynolds, 2006), responds rapidly to environmental variations (Reynolds, 2006), its size structure can affect the food web (Brander & Kiørboe, 2020) and plays a key role in biogeochemical processes (Litchman et al., 2015). Due to these features, phytoplankton is one of the key BQEs required by the WFD (Birk et al., 2012). Its biomass, abundance, community structure and diversity are usually employed as metrics for ecological status assessment of lakes (Katsiapi et al., 2016; Laplace-Treyture & Feret, 2016), rivers (Wu et al., 2012) and marine/coastal environments (Devlin et al., 2009; Spatharis & Tsirtsis, 2010).

The standardised method used in the WFD to identify phytoplankton and estimate its abundance and biovolume is based on light microscopy (CEN, 2006). This method is tedious and requires wide taxonomic expertise. Microscopy-based assessment is also subject to potential bias due to the difficulty, or even inability, to identify very small species (e.g., some picocyanobacteria), as well as the presence of cryptic or very rare taxa (Jackson et al., 2014; Kermarrec et al., 2013). Methods based on molecular techniques using high-throughput sequencing and short DNA sequences, such as DNA metabarcoding, have provided an alternative to overcome these issues (Baird & Hajibabaei, 2012). Since the mid-2010 s, a large number of studies using DNA metabarcoding have been conducted to describe phytoplankton communities in freshwater (e.g., Banerji et al., 2018; Fabrin et al., 2020; Lin et al., 2023) and marine (e.g., Catlett et al., 2023; Marinchel et al., 2023; Yoon et al., 2016) habitats, or for monitoring purposes (e.g., Hanžek et al., 2021; Nicolosi Gelis et al., 2024). Several studies compared the differences between microscopy and DNA metabarcoding (e.g., Abad et al., 2016; Bilbao et al., 2023; Nicolosi Gelis et al., 2024; Wang et al., 2022). An important challenge when targeting the entire phytoplankton community composition is its polyphyletic nature, which makes it difficult to select molecular markers with good coverage. One solution is to target gene sequences present in both prokaryotes and eukaryotes, e.g. the plastid 16S rRNA (Eiler et al., 2013; Kirkham et al., 2013; Nübel et al., 2000), or 23S (Sherwood & Presting, 2007; Steven et al., 2012; Yoon et al., 2016). The comparative studies between molecular and microscopic methods show a moderate overlap, with ca. 10 % of shared species (Andersson et al., 2023; Nicolosi Gelis et al., 2024), each method having its specific bias preventing a perfect view on the total diversity. On one hand, the incompleteness of reference libraries, whatever the library and marker considered, means that only a small amount of phytoplankton sequences are covered (Tzafesta et al., 2022). On the other hand, microscopy struggles to detect small or hardly recognizable cells, particularly picoalgae that can, however, largely contribute to some lake phytoplankton communities (Somogyi et al., 2020; Zhong et al., 2013). Despite its biases, metabarcoding provides a more exhaustive characterization of the true diversity of phytoplankton, which is of major interest for ecosystem monitoring.

In addition to the taxonomic aspect, phytoplankton communities are also often studied through a trait-based approach, which is also implemented in some cases as bioassessment tools (Padisák et al., 2006; Salmaso et al., 2015). Traits refer to any measurable features on the individual level (Violle et al., 2007) which can be considered as the manifestation of the way particular individuals adapt to specific environments, enlightening a direct link between the selective factors and the organisms (Salmaso et al., 2015). Individual traits may have important indicator value and different types of functional groups, based on specific criteria, have also been developed for phytoplankton, among which the Morphologically Based Functional Groups (MBFG; Kruk et al., 2010) and the Reynolds' Functional Groups (RFG; Padisák et al., 2009; Reynolds et al., 2002). These functional groups are based, respectively, on morphological characteristics (MBFG) or on phenological, ecological and functional characteristics (RFG). Although DNA metabarcoding and trait-based approaches are two cutting-edge fields in ecological research and bioassessment, few studies yet focus on merging these approaches and enhance the potential of metabarcoding beyond taxonomic identification (Hanžek et al., 2021).

This study's aim was to provide answers to the elements outlined above for this field of biological monitoring. This purpose was possible using a reference barcoding library (Phytool v3) containing taxonomic information on phytoplankton 23S rRNA barcodes annotated with trait information. Our goal was to (i) integrate trait assignment into the bioinformatic pipeline so that traits can be directly assigned to amplicon sequence variants (ASVs) and (ii) estimate the relevance of trait-based assignment in assessing the structure of environmental communities. To complete the first objective, an *in silico* validation test on the Phytool v3 library was carried out. We hypothesised that a higher proportion of sequences would be assigned to traits than at fine taxonomic resolutions (i.e., genus or species). We also hypothesised that traits exhibiting good phylogenetic signals (i.e., when good congruence between the values of a trait and the genetic distances between sequences is observed) would have good assignment of sequences. For the second objective, an environmental assessment was carried out. We hypothesised that assigning traits to ASVs from environmental samples should yield meaningful and interpretable results on how communities are structured in the different lakes. To do this, four large alpine lakes in France (Aiguebelette, Annecy, Bourget and Geneva lakes) were sampled every month for a year, and the dynamics observed with traits and taxonomic data were compared.

2. Materials and methods

2.1. Database description

Here, the third version of the Phytool (v3) reference library is presented and tested. Phytool v1 (Canino et al., 2021) was composed of sequences belonging to algal taxa mainly for two marker genes, 16S and 23S rRNA. The data sources included the Silva_138.1 (Quast et al., 2013), PR2 (Guillou et al., 2013) and Phytoref (Decelle et al., 2015) databases for 16S rRNA, and Silva_138.1 and µgreen-db (Djemiel et al., 2020) databases for 23S rRNA. Additionally, each sequence was taxonomically curated in Phytool v1. Phytool v2 included an additional 13 barcodes for 16S and 25 barcodes for 23S. These new barcodes come from phytoplankton cultures of the Thonon Culture Collection (Rimet et al., 2018) and were sequenced in the study of Canino et al. (2023). For Phytool v3, the focus has been on 23S rRNA sequences and to perform sequence annotation with traits. The reference library Phytool v3 (Rimet et al., 2024) is freely available at the following link: https://doi.org/10.57745/TIRNFD.

Data sources to carry out the morphological and ecological trait annotations come from open access trait databases for freshwater algae (Laplace-Treyture et al., 2021; Rimet & Druart, 2018), WoRMS (WoRMS Editorial Board, 2023) and the literature. Six traits divided into 56 categories and two functional groups were gathered in Phytool v3. Qualitative traits were described according to a suite of nominal categories, for example: "autotrophic" and "mixotrophic" for the trait "Nutrition". Quantitative traits were expressed either by ordinal categories describing a gradient, for example: a gradient of chloroplast number or directly by quantitative value (e.g., organic carbon content ratio). Qualitative traits were coded using a disjunctive approach (i.e., the category is used (=1) or not used (=0) by the taxon). The description of traits is available in Supplementary Materials (Table S1) and further information can be found in the Phytool v3 library.

The trait "Size" is based on the classically recognized size classes of planktonic algae (e.g., Lévêque, 2001), completed with the "macroalgae" class. "Habitat" and "Morphology" traits are based on the work of Rimet & Druart (2018), Laplace-Treyture et al. (2021), WoRMS database, additional literature sources (Table A.1) and expert knowledge of the authors of this study. The trait "Nutrition" follows the definitions proposed by Lwoff et al. (1946). The trait "Organic carbon content ratio" is based on the carbon ratio proposed by Wetzel & Likens (2000), which enables the calculation of phytoplankton carbon weight from its biomass. The traits "Pigments, toxins" is based on the work performed by Laplace-Treyture et al. (2021). Finally, the functional groups of

Padisák et al. (2009) and Kruk et al. (2010) are given for those freshwater taxa mentioned in these two publications (marine and soil algae being excluded).

An alignment of the 23S sequences is given in Phytool v3. Alignments were first carried out for each phyla with Muscle in Seaview v4 (Gouy et al., 2010), and then they were merged. Sequences which were too short, or those presenting large introns (e.g., Charophyta), or those which were misaligned, were not included in the alignment.

2.2. An in silico validation test of the Phytool v3 reference database

In order to assign ASVs directly to traits and compare the success of their assignment by comparison with taxonomic assignment, we set up a two-stage procedure. In the first step, a reference FASTA file for each trait was created by combining information on that trait and the 23S alignments from the Phytool v3 library, so that each database contains reference sequences and their corresponding category for the given trait. Sequences with unavailable trait information were removed. In a second step, the performance of the functional assignments was estimated by using a leave-one-out cross-validation (LOOCV) procedure. To achieve this, each reference sequence in the database was assigned to a subset of the reference trait database from which the sequence in question was removed. This procedure was performed with all trait databases and taxonomic levels. Finally, the assigned trait class to a given sequence was compared with the original class referenced in the database. The same comparison was carried out for taxonomy. Correct assignments, false assignments and unassignments were reported. Taxonomic assignment was carried out with the *assignTaxonomy()* function from the R-package *dada2* (Callahan et al., 2016) that uses the Ribosomal Database Project (RDP), a naïve Bayesian classifier method (Wang et al., 2007), and a minimum bootstrap of 75 %. Two traits; carbon content and Anatoxin-a(S) were removed for subsequent analyses. Carbon content is the only trait with continuous values and thus our procedure of validation is not adapted for it. The trait Anatoxin-a(S) contains only zero values for all barcodes, thus it is not relevant for the *in silico* test.

2.3. Do genetically similar taxa have similar traits and does this explain a higher trait assignment success?

We expected some traits to have higher assignment success than others, depending on their level of congruence with the genetic distance between sequences. In order to assess the congruence between genetic similarity and traits similarity, a matrix of pairwise distances between the barcodes was computed using the *dist.gene()* function of the R-package *ape* (Paradis & Schliep, 2019). To visualize the relative position of barcodes in the genetic space, a Principal Coordinate Analysis (PCoA) was then carried out on the distance matrix (Euclidean distance) using the *wcmdscale()* function of the R-package *vegan* (Oksanen et al., 2019). Ordination plots were generated for each taxonomic level and traits, grouping data points based on the taxonomy and trait classes (Fig. S1). Significance between groups based on taxonomy or traits was tested with a permutational multivariate analysis of variance using the genetic distance matrix and the *adonis()* function of the R-package *vegan.*

2.4. Test of the Phytool v3 database on environmental samples

In order to assess the relevance of the trait-based sequence assignment compared to taxonomic assignment, we assigned environmental sequences coming from lake samples. We used the environmental dataset of Nicolosi Gelis et al. (2024), which included samples (n = 61) collected in 2021 from four large-alpine lakes: Aiguebelette, Annecy, Bourget and Geneva. The detailed methodology for field sampling, DNA extraction, amplification and sequencing of the 23S rRNA marker gene is given in Nicolosi Gelis et al. (2024). Briefly, samples were taken every month or two weeks in 2021 with an integrated IWS water sampler between 0–18 m. Then 250 ml of water sample was filtered in the laboratory on open filters with a porosity of 0.45 µm (MF-Millipore®, cellulose nitrate membrane filter). The DNA extraction was carried out with a Nucleospin Soil kit (Macherey-Nagel) and the PCR amplification of the UPA region of the 23S was carried out by using ECLA23S_F1 (5′-ACAGWAAGACCCTATGAAGCTT-3′) ECLA23S_R1 (5′- CCTGTTATCCCTAGAGTAACTT-3′) primers (Canino et al., 2023), following the protocol described in Nicolosi Gelis et al. (2024). PCR products were sent for sequencing to the PGTB platform (Plateforme de Genomique et Transcriptomique, Bordeaux, France), using MiSeq technology (Illumina, SY-410–1003) and the v3 reagent kit (2 × 250 bp).

The demultiplexed MiSeq reads of the 61 environmental samples were analysed with the DADA2 pipeline (Callahan et al., 2016) by adapting the settings to analyse phytoplankton based on the 23S marker (Canino et al., 2023). Primers were removed from forward and reverse reads with cutadapt 2.9 (Martin, 2011). The quality profiles of reads were then verified, and forward and reverse reads were truncated to 220 and 180 nucleotides, respectively, in order to remove poor quality nucleotides towards the ends. Truncated sequences were filtered out with a criterion of 0 ambiguities (“N”) and a maximum of expected errors (maxEE) of 2 using the *filterAndTrim()* function from the R-package *dada2* (Callahan et al., 2016). An error model was executed using the *learnErrors()* function and showed that estimated error rates fit the observed rates well, and the error rates decreased with increased quality. Reads were dereplicated into individual sequence units using the *derepFastq()* function. ASVs were then selected based on the error rate models and paired reads were merged into one sequence using the *dada()* function with default options before being merged. Finally, chimeras were removed using the *removeBimeraDenovo()* function. Taxonomic and trait assignment of ASVs were performed using the Phytool v3 reference barcode library presented in this study and using the naïve Bayesian classifier implemented in the R-package dada2 (*assignTaxonomy()* function), with a minimum bootstrap confidence value of 75 %.

The assessment of how phytoplankton communities are characterized from trait-assigned metabarcoding data give interpretable results compared to classical taxonomic assignment of metabarcoding data, was evaluated with non-metric multidimensional scaling (NMDS) analyses, using the *metaMDS()* function of the R-package *vegan* using Bray-Curtis dissimilarity. Only for the NMDS analyses, in order to handle differing read numbers per sample, data was rarefied to the lowest read number per sample (36,705) and singletons were additionally removed. The *anosim()* function of the vegan package in R was used to perform analysis of similarities (ANOSIM) to test if the ASV, species, MBFG and RFG assemblages differed significantly between the lakes.

3. Results

3.1. Content and accessibility of the annotated reference library Phytool v3

After an alignment and a taxonomic curation, the reference library Phytool v3 contained 1329 unique sequences. These sequences corresponded to 816 species and 467 genera, distributed heterogeneously among 14 phyla (Fig. 1). Bacillariophyta gathers 57 sequences, Cercozoa 6 sequences, Charophyta 90, Chlorophyta 240, Cryptophyta 49, Cyanobacteria 374, Euglenozoa 210, Glaucophyta 4, Haptophyta 9, Miozoa 6, Ochrophyta 103, Prasinodermatophyta 6, Rhodophyta 171 and Tracheophyta 4 sequences. Trait annotation is complete for a large majority of sequences except for e.g. the two functional groups (MBFG, RFG), which are available only for freshwater taxa representing 45.1 % of the sequences. Pigment and toxin traits were lacking in Glaucophyta (5 barcodes), Prasinodermatophyta (6 barcodes), Rhodophyta (197 barcodes), Tracheophyta (4 barcodes) and Cercozoa (8 barcodes), and to a lesser extent in Ochrophyta (117 barcodes from the total 220) and Charophyta (93 barcodes from the total 130). Detailed annotation completeness is available in the reference library Phytool v3.

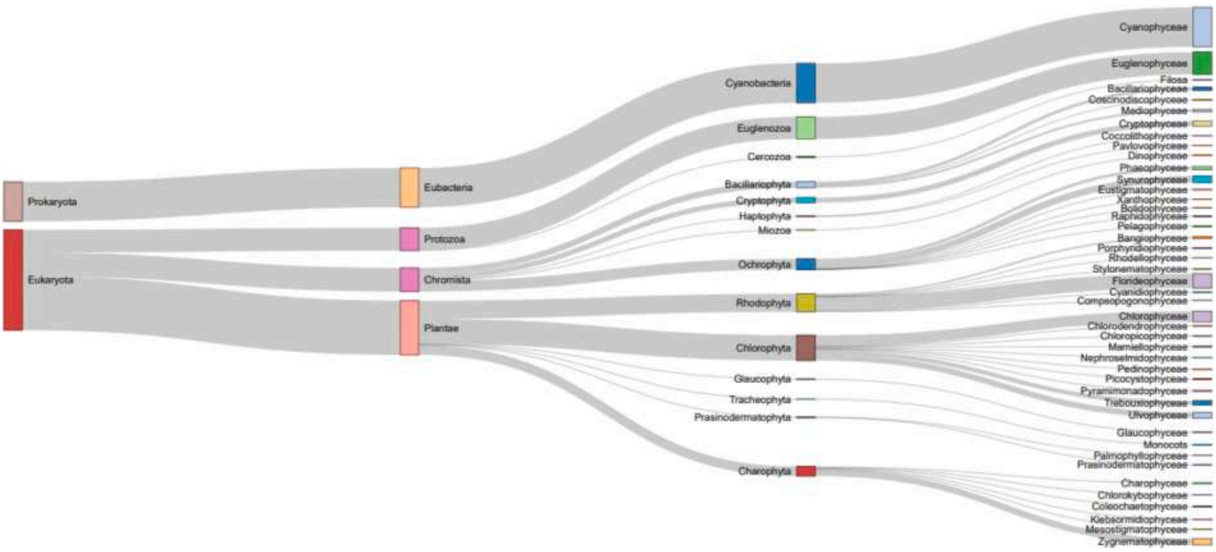


Fig. 1. Sankey diagram showing the number of sequences for the different taxonomic groups in the Phytool v3 reference library.

3.2. *In silico validation tests of the reference library Phytool v3*

During the *in silico* validation tests, each sequence was assigned using taxonomic and trait reference tables that excluded the analysed sequence. This analysis was carried out for all sequences contained in Phytool v3 (n = 1329). The number and the percentage of sequences correctly or incorrectly assigned to the taxonomy and to the different traits and those remaining unassigned were then estimated. Regarding taxonomy, false assignments were 20 % at species level, 10 % at genus level and between 1 % and 5 % at higher taxonomic levels (Fig. 2). The proportion of correct assignments was 40 % for species, 62 % for genus and already above 90 % from the class level. For the traits, correct assignments varied between 79.8 % (RFG) and 99.2 % (heterocyte) and false assignments varied from 0.34 % (heterocyte) to 8.42 % (mucilage). The proportion of unassigned sequences was the lowest for Heterocyte (0.43 %) and the highest for RFG (13.2 %).

3.3. *Do genetically similar taxa have similar traits and does this explain a higher trait assignment success?*

PCoAs were performed using Kingdom and Phylum taxonomic levels and traits, are shown on Fig. 3. and Fig. S1. After performing the PERMANOVAs, we first found that the number of classes within a trait has significant and positive effect on the R^2 values ($r = 0.66$, $p < 0.001$), thus explaining a higher proportion of variance in the data. On the contrary, the number of classes within a trait negatively affects the percentage of correct assignments ($r = -0.35$, $p < 0.01$). Thus, the relationship between PERMANOVA R^2s and the correct assignments is not clear at first sight. Even when we looked for the relationship between R^2 and successful assignment percentages considering only traits with two classes (0 and 1), only weak and marginally positive ($r = 0.23$, $p = 0.09$) relationship was found.

3.4. *Application of trait assignment to environmental samples*

Based on the 61 samples from the four large-alpine lakes, a total of 3090 ASVs for 3,533,077 reads were recovered. From these total ASVs, 1794 (58.1 %) were assigned to 10 algal phyla: Cyanobacteria, Bacillariophyta, Cryptophyta, Haptophyta, Ochrophyta, Chlorophyta, Rhodophyta, Charophyta, Euglenozoa and Tracheophyta, the other being unassigned to this taxonomic level. The percentage of successfully assigned ASVs decreases with the taxonomy level and only 584 ASVs (18.9 %) were assigned to species level (Fig. 4A). When considering the read numbers (abundance), higher assignment efficiency could be achieved. Indeed, 45.9 % of the reads were assigned to species and more than 95 % to Class, Phylum and Kingdom. Assignment efficiency of ASVs differed depending on the trait considered: from 49.4 % (number of plastids) to 98.3 % (Heterocyte) of the ASVs were assigned to traits (Fig. 4B). Similarly, to the taxonomic assignment, considering read number, the assignment efficiency increased with 12.9 % on average.

The rarefied dataset without singletons that was used for the NMDS analyses contained 2442 ASVs compared to the original 3090 ASVs. The four independent NMDSs based on ASV, species, RFG and MBFG data, showed a distinction in samples based on the origin lake (Fig. S2). The significance value of the ANOSIM test was less than 0.05, meaning that the null hypothesis can be rejected, and therefore that samples are statistically different according to grouping (the origin lake), regardless of data type. In addition, the R statistics was positive for all ANOSIM tests, indicating greater similarity within lakes and therefore greater dissimilarity between lakes: the strongest R statistic was obtained in the case of ASV data ($R = 0.55$) and lower values of 0.30, 0.28 and 0.16 for species, RFG and MBFG, respectively. The calculation of pairwise Euclidean distance between barycenter of lakes displayed in NMDSs showed that similarity between lakes was more or less important depending on the type of data considered (Fig. 5). Overall, pairwise Euclidean distances were lower (meaning greater similarity) between Lakes Aiguebelette, Annecy and Bourget than for Lake Geneva for the four data types. When considering data type, pairwise Euclidean distances were lowest for MBFG data and gradually increased for species, RFG and ASV.

4. Discussion

Assessment of the ecological status of lake ecosystems is often based on the identification of sensitive and/or tolerant species to a given pressure, and their integration into the calculation of biotic indices (e.g., Brettum, 1989; Laplace-Treyture & Feret, 2016). Metabarcoding provides access to diversity, much of which may be hidden to conventional approaches to diversity characterization (e.g., microscopy). However, assigning a fine taxonomy (e.g., genus or species name) to a sequence can sometimes be difficult, notably due to the lack of completeness of the reference libraries used to realise the taxonomic assignment (Weigand et al., 2019). An alternative is to use traits as a substitute to the taxonomy in sequence assignment. The interest to use traits come from the fact that they provide clearer mechanistic links to ecosystem functioning, because they are the main properties by which organisms influence ecosystem processes (Gagic et al., 2015; Petchey & Gaston,

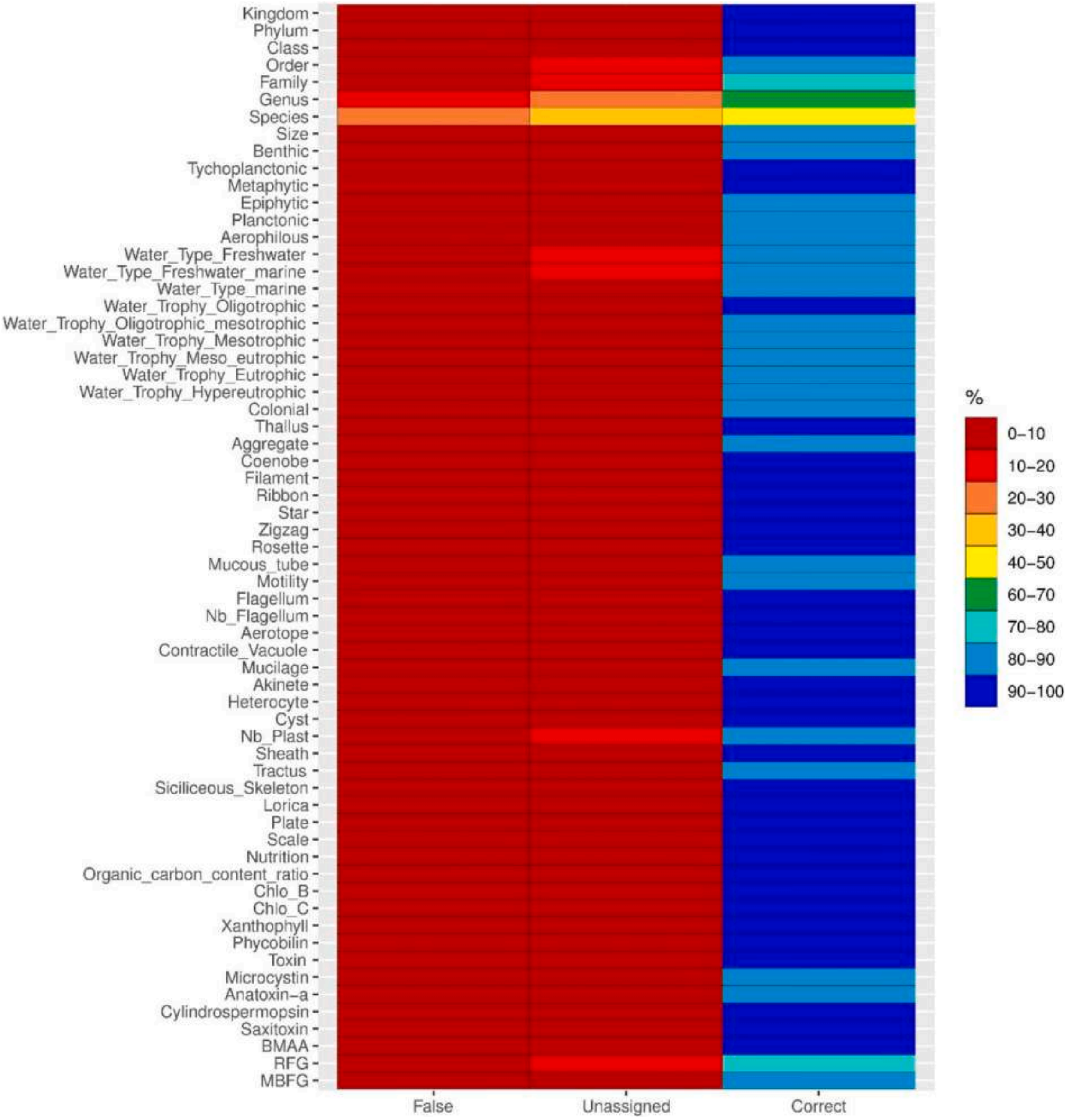


Fig. 2. Proportion of incorrectly (false) and correctly assigned, and unassigned sequences for the seven taxonomic levels and for the 56 traits.

2006). In this study, we address this question by compiling the Phytool v3 reference library with both taxonomy and trait information. Using Phytool v3, we have shown that traits can be assigned to sequences with high reliability and better coverage than assignment to a fine-taxonomy level.

4.1. *Do genetically similar taxa share similar traits?*

In our study, we found that all traits are conserved in the phylogeny of phytoplankton, but to different degrees. Traits can be grouped into two categories according to their maintenance along the phylogeny. Some traits are highly conserved in the phylogeny, while others are conserved to a lesser extent.

The good match with phylogeny concerned the possession of heterocytes and akinetes, which are found only in the Nostocales, a monophyletic order of Cyanobacteria (Komárek & Johansen, 2015). Taxa with these traits have the ability to fix dissolved atmospheric nitrogen with the heterocytes and form resting cells (akinetes), which transiently accumulate storage compounds to survive under harsh environmental conditions (Adams & Duggan, 1999). Another example is the presence of a siliceous skeleton. This trait is only found in Bacillariophyta (frustule) and Ochrophyta (scale), two monophyletic phyla of the Chromista kingdom (Kociolek & Williams, 2015; Nicholls & Wujek, 2015), but also in *Paulinella* sp., a photosynthetic amoeba (Nomura & Ishida, 2016). Similarly, plates are only observable in Dinophyta which constitute the theca composed of cellulose, and whose morphology is an essential criterion for the identification and taxonomy of this phylum (Carty & Parrow, 2015).

Several traits related to habitat-preference are also well conserved in the phylogeny. Indeed, we observe that genetically close taxa have similar habitat- and ecology-preference. This echoes the hypothesis that, according to the phylogenetic niche conservatism, evolutionary lineages tend to maintain their ancestral ecological niche (Wiens, 2004). The transition from marine to freshwater habitat was a major evolutionary

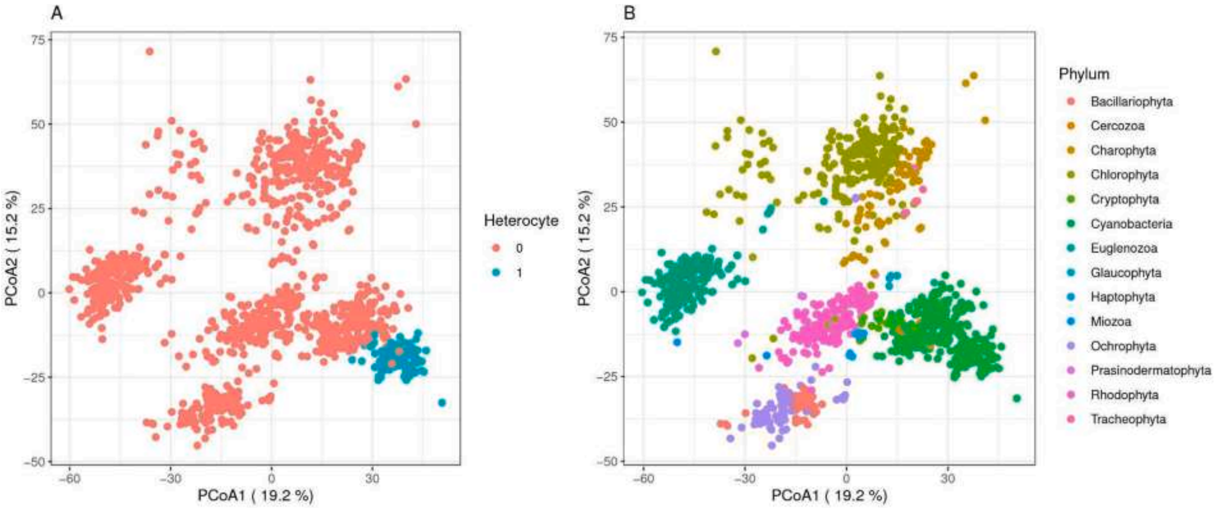


Fig. 3. Phytoplankton community structure represented by the projection of ASVs (dots) on the first two axes of the PCoA based on the pairwise genetic distance between ASVs. Dots are coloured according to (A) heterocyte trait (PERMANOVA, $R^2 = 0.08$) and (B) phylum (PERMANOVA, $R^2 = 0.49$).

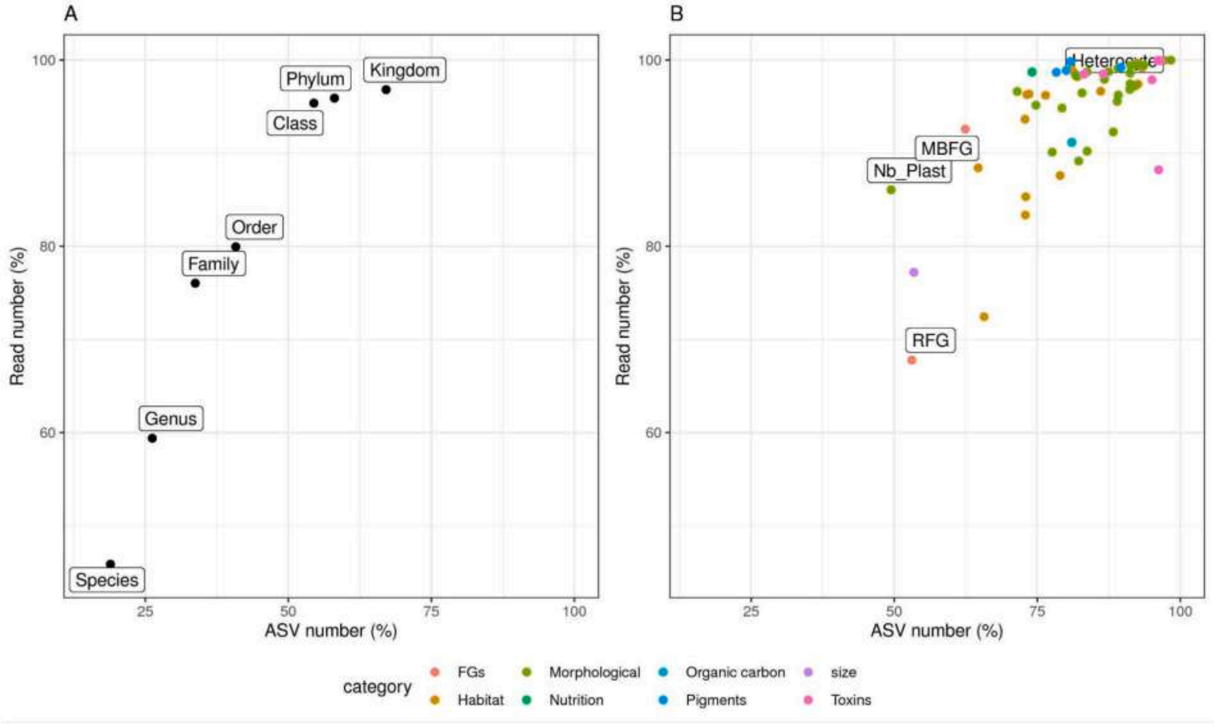


Fig. 4. Proportion (%) of the number of ASVs and read numbers successfully assigned to the different taxonomic levels (A) and traits (B). Trait categories are indicated by colours and the position of the traits with the minimal and maximal ASV number coverage (number of plastids (Nb_Plast) and heterocyte, respectively); and the two functional groupings (MBFG, RFG) used in this study are indicated by labels as well.

milestone for several organisms, including algae (Dittami et al., 2017). As a result, some clades are restricted to the marine or the freshwater realm, an observation that we have also made in our data. For instance, Bolidophyceae is exclusively found in marine habitats (Kuwata et al., 2018). Haptophyta are common and highly diversified in marine environments, with a few exceptions to freshwater lineages which were recently sequenced (Shalchian-Tabrizi et al., 2011). On the contrary, Chlorophyta are mostly freshwater organisms (Leliaert et al., 2012) except for a few classes (e.g., Ulvophyceae) that are more common in marine environments (McAvoy & Klug, 2005; Romano et al., 2003). Diatoms are found in both environments (Vanormelingen et al., 2008). This cosmopolitan distribution of diatoms was made possible by a few clades that managed the evolutionary transition from the marine to the freshwater environment, where they rapidly diverged (Nakov et al., 2019). However, some clades are specifically adapted to marine or freshwater environments (Vanormelingen et al., 2008). Traits related to habitat life form (benthic, metaphytic and epiphytic) also showed taxonomic homogeneity at different levels and extent. For instance, diatoms are present in both benthic and pelagic habitats, and habitat shifts are rare, implying niche conservatism in large clades (Nakov et al., 2015). Rhodophyta taxa are mostly benthic (e.g., Sheath & Vis, 2015) except for five species out of the 156 in our database. Within the Charophyta, the habitat life form is conserved at the class level, as can be seen in the Charophyceae and Coleochaetophyceae, which are benthic

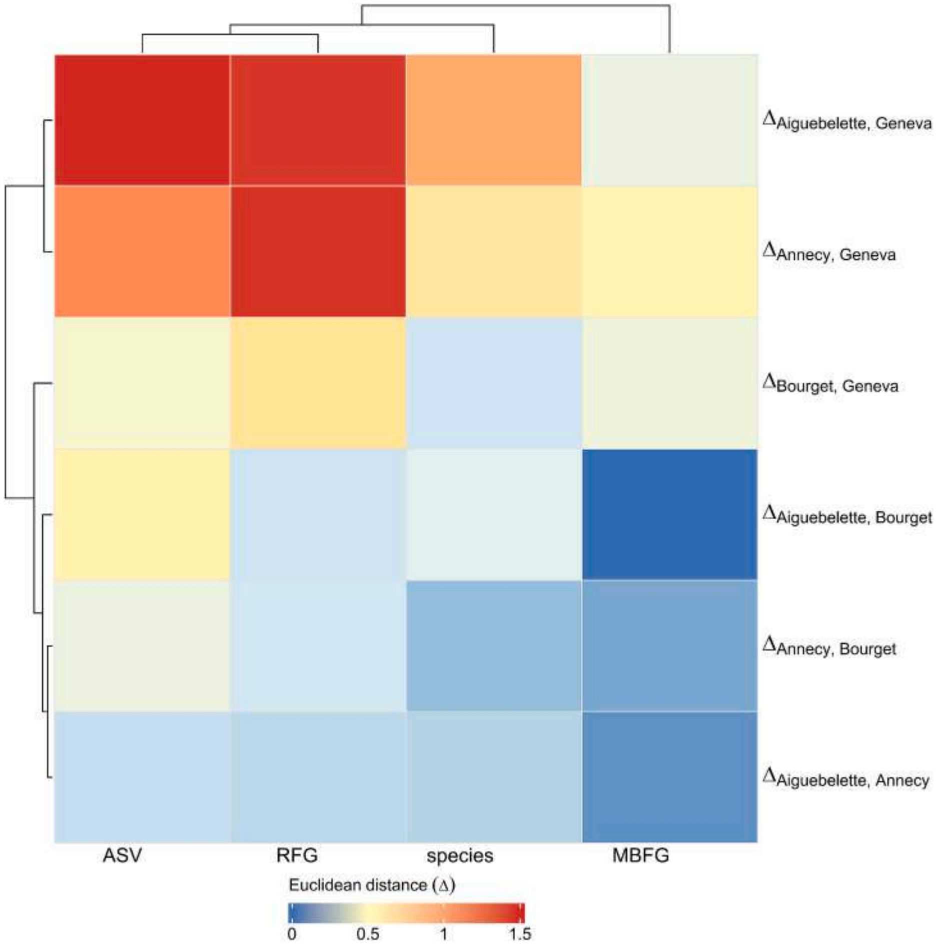


Fig. 5. Heatmap showing the Euclidean distances (Δ) between each pair of lakes (Aiguebelette, Annecy, Bourget, Geneva) for the four types of data: ASV, species, Reynold's Functional Groups (RFG) and Morphology-Based Functional Groups (MBFG). Euclidean distances were calculated from the scores of barycenters of lakes on the factorial plan of the NMDSs.

or epiphytic classes. In contrast, for the Zygnematophyceae, another class of Charophyta, the habitat life form is conserved at a lower taxonomic rank, the order with the Desmidiales being planktic or metaphytic, while the Zygnematales taxa are mainly benthic. Phylogenetic conservatism was also observed for other traits related to habitat life form (e.g., trophic status, aerophilic), nutrition, morphology (e.g., number of chloroplasts, colony, motility, flagella), pigment type and organic carbon content ratio.

While the traits mentioned above are well conserved in the phylogeny, a lesser degree of phylogenetic conservatism is observed for other traits. One of these traits is size. The size is a well-observable and easily measurable trait of significant ecological interest, for example in grazing resistance (Lürling, 2021), nutrient acquisition (Litchman et al., 2007) and sinking regulation (Padisák, 2003). Regarding the classical size classes used in this study, some clades include exclusively macroalgae (e.g., Phaeophyceae, Charophyceae, Ulvophyceae, Florideophyceae), microalgae (e.g., Euglenozoa) or picoalgae (e.g., the unicellular cyanobacteria Synechococcales). Other groups contain taxa with different size classes. For instance, within the Chlorophyta, size was only homogeneous at family level (e.g., Coccomyxaceae and Chlamydomonadaceae include exclusively nanoalgae). In the same way, but at finer taxonomic level, the family Volvocaceae includes genera of both nano- and microalgae. The absence of good phylogenetic conservatism of size in diatoms (Nakov et al., 2014) has also been observed in Phytool v3.

Like body size, MBFGs have shown less phylogenetic conservatism, characterized by variable phylogenetic homogeneity between groups. For instance, MBFG group I includes small-celled organisms with a high surface-to-volume ratio, belonging to the phyla Chlorophyta, Cyanobacteria, Cryptophyta. In contrast, MBFG group II contains only Synurophyceae and some Coccolithophyceae, which are flagellated algae with siliceous exoskeletal structures. The MBFGs were constructed based on the assumption that morphological traits of phytoplankton individuals reflect their requirements and functionality related, for example, to how they acquire nutrients, avoid grazing or sedimentation (Kruk et al., 2010). In contrast to MBFGs, RFGs gather taxa that can have various morphological traits. Indeed, RFGs are based on a phytosociological approach, defining species associations that are occupying similar ecological niches in the same environment. Despite this approach to group construction, we observed that genetically close taxa tend to belong to the same functional groups, but to a much lesser extent for most other traits.

4.2. Impact of trait similarity for genetically similar taxa on assignment success

The assignment success results obtained by the *in-silico* (assignment of Phytool sequences on the Phytool reference library itself) and *in-situ* (lake samples) approaches were comparable. Indeed, whatever the approach, there was a progressive decrease in assignment success from higher taxonomic ranks (i.e., class to kingdom) to lower taxonomic ranks (i.e., genus and species). Insofar as higher taxonomic ranks are relatively uninformative for ecological studies, the trait-based approach is of real interest. However, it is important to keep in mind that while assignment success was high for some traits (e.g., akinete, heterocyte, plate, lorica, scale, thallus, and filament); this is not the case for others (e.g., RFGs) whose assignment success is lower.

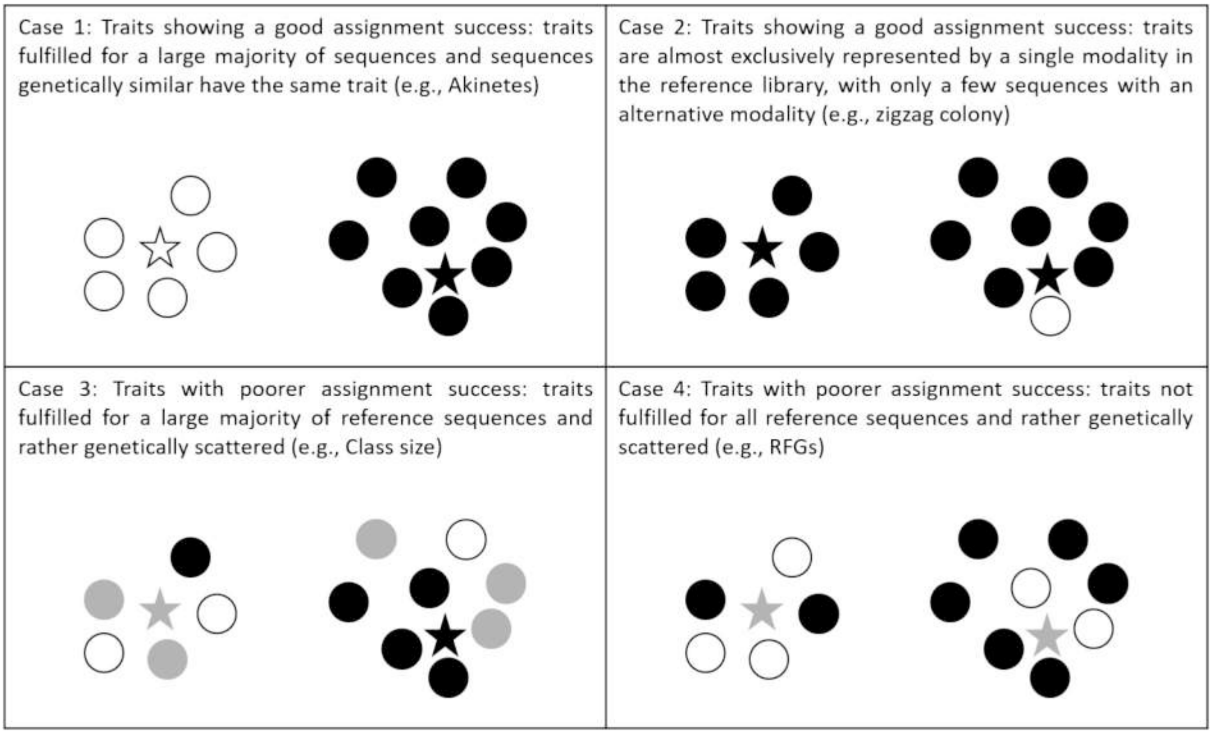
The taxonomic and trait assignment of ASVs from lake samples was carried out by using the Ribosomal Database Project (RDP) classifier, a naïve Bayesian classifier (Wang et al., 2007). The RDP classifier is based on the SeqMatch tool, a k-nearest-neighbor (K-NN) classifier (Cole et al., 2005), which uses a word-matching strategy not requiring alignment to determine the percentage of shared eight-character words between a query and members of a database of sequences. This tool assigns the query sequence to the lowest taxonomic rank, which includes the highest scoring neighbouring sequences of the reference library. Sequence assignment with this approach is therefore relied on two factors i) the sequence similarity between the references and the query, and ii) the number of neighbours in the reference database. Four cases are presented below (Fig. 6), illustrating the impact of these two factors, together with the completeness of the trait database, on assignment success.

The first case corresponds to the best assignment success encountered in our study. Here, the good assignment success lies in the fact that traits are attributed to a large majority of sequences and that genetically similar sequences have the same traits. Traits fulfilling this condition include: akinetes, heterocytes, siliceous skeleton, plates and scales. In the previous section (section 4.1), we emphasized that these traits are restricted to particular algal clades, and that they have a sufficient number of reference sequences and organisms that have their trait fulfilled. This enables an efficient assignment with the RDP classifier. For instance, for these traits, even if an environmental sequence in our study lakes did not exactly match a reference sequence, since several neighbours share the same trait (e.g., presence of scales), then the trait can be easily assigned to it.

The second case illustrates traits whose assignment is good, but for which the whole sequences present a single modality (i.e., absence of trait) in the reference database, apart from a few sequences that have no information on the trait. Rosette colonies, zigzag colonies, mucous tubes colonies are traits fulfilling the conditions of this second trait category. For these traits, only four or five sequences out of 1996 sequences in Phytool, present these particular colony types. It is therefore clear that in the vast majority of cases, the environmental sequences of the lakes studied were identified as not having this type of colony, since the vast majority of the sequences in the Phytool do not have this type of colony. However, it is possible that there is an error rate in identification, due to a “mass effect” of reference sequences not having this type of colony compared to the rare reference sequences having this type of colony.

In the third case, traits have a poor assignment success rate, even though they are present in a large majority of reference sequences in the Phytool library and are rather genetically scattered. Only the trait size class is classified in this group. While some large clades were homogeneous in size class, with only one class assigned, other clades were more heterogeneous in size class. Chlorophyta and Diatoms are two important algal classes in the ecology of *peri*-alpine lakes, including those studied (Frossard et al., 2022; Jacquet et al., 2022; Rimet et al., 2022). The size of taxa in these algal classes are heterogeneous even at lower taxonomic ranks (see section 4.1 of the discussion). This observation may explain why assignment to size class only reached 53 % of sequences (and 77 % of reads) from the lakes studied.

The last case brings together traits with a low assignment success rate, which are missing for many reference sequences of the Phytool library and are rather genetically scattered. Here also, only one trait (RFG) is classified in this group. While there is a general tendency for neighbouring sequences to belong to the same functional group (confirmed statistically), in detail, things are less clear-cut. Indeed, neighbouring sequences may belong to different functional groups (a given functional group can gather taxa belonging to various taxonomic groups). In addition, there are many reference sequences, such as those belonging to marine taxa, for which the functional group is not indicated. Therefore, for many environmental sequences in our study lakes, which do not exactly genetically match the freshwater sequences in Phytool, but are closer to the marine sequences, the RDP classifier was unable to assign them a functional group.



Case 1: Traits showing a good assignment success: traits fulfilled for a large majority of sequences and sequences genetically similar have the same trait (e.g., Akinetes)

Case 2: Traits showing a good assignment success: traits are almost exclusively represented by a single modality in the reference library, with only a few sequences with an alternative modality (e.g., zigzag colony)

Case 3: Traits with poorer assignment success: traits fulfilled for a large majority of reference sequences and rather genetically scattered (e.g., Class size)

Case 4: Traits with poorer assignment success: traits not fulfilled for all reference sequences and rather genetically scattered (e.g., RFGs)

Fig. 6. Diagram showing the four cases of impact of trait similarity for genetically similar taxa on assignment success. Circles correspond to reference sequences in the Phytool library and stars represent query sequences (i.e., environmental sequences). The black and white colours correspond to a modality for a given trait and grey colour corresponds to unknown trait modality (for reference sequences) or unassigned trait (for query sequence).

4.3. Application of the trait-based assignment approach on environmental samples

We hypothesized that the direct assignment of environmental ASVs to bio-ecological traits would provide meaningful and interpretable results on how communities are structured in the four large French *peri*-alpine lakes. The value of direct trait assignment is demonstrated by a comparison with a taxonomy-free approach (no sequence assignment) and with classical taxonomic species assignment. The advantages and disadvantages of the three approaches are highlighted according to three criteria related to phytoplankton community structure and bioindication ability: i) beta diversity, ii) assignment success, ii) ecological information (Table 1).

Firstly, compared with species- and trait-based (MBFGs, RFGs) approaches, the taxonomy-free approach (ASV) provides the greatest discrimination between lakes, expressed in terms of dissimilarity in community composition (i.e., beta diversity). One possible reason for the greater beta diversity with the taxonomy-free approach than with the species-based approach is the ability to take into account the hidden diversity of certain phytoplankton groups. For example, while picocyanobacteria may dominate lake phytoplankton communities in terms of read number (Li et al., 2019), a large diversity of genotypes is not detected by microscopy (MacKeigan et al., 2022; Xiao et al., 2014), resulting in a diversity that is not formally described taxonomically. Such results were observed in the lakes of our study, where phytoplankton communities were dominated by picocyanobacteria, which presented numerous genotypes (Nicolosi Gelis et al., 2024). Other microalgae groups, such as diatoms, also harbour significant diversity of genotypes with a high degree of endemism (Mackay et al., 2006; Rimet et al., 2023) visible when diversity is assessed by the taxonomy-free approach, but not visible with the species-based approach (Tapolczai et al., 2021). These findings may explain the greater beta diversity observed with the taxonomy-free approach. In addition, given that different species can have similar traits, this may reduce the dissimilarity between samples and thus explain the lower beta diversity observed with the trait-based approach.

It is also interesting to note, in the comparison between the species- and trait-based approaches, that the dissimilarity of communities between lakes can be greater or lesser depending on the trait considered. Indeed, even if different species can have similar traits, and the species-based approach should give a higher beta diversity than trait-based approach, another parameter comes into play, namely assignment success. Assignment success is much lower for the species-based approach than for the trait-based approach. The resulting loss of data, which is greater for the species-based approach, may explain the lower beta diversity between lakes for this approach than certain traits (i.e., MBFGs). Reference libraries are far from complete, particularly for microalgae (Weigand et al., 2019). Completing libraries is of crucial importance if a species-based approach is to be chosen for metabarcoding monitoring of large French *peri*-alpine lakes (Nicolosi Gelis et al., 2024). For instance, important genera such as *Kephyrion*, *Dinobryon* are not present in Phytool v3 library, even though they may dominate the algal biomass in the oligotrophic lakes Annecy and Aiguebelette (Rimet et al., 2022).

Finally, each of the three approaches carried a substantial amount of ecological information. Concerning the taxonomy-free approach, its relevance for the ecological assessment of rivers based on benthic microalgae and the development of biotic indices has already been tested (Apothéloz-Perret-Gentil et al., 2017; Tapolczai et al., 2018, 2021). While in this approach, no prior ecological knowledge is (usually) linked to ASVs, it needs to estimate their ecological profile to calculate biotic indices. At this step, we can mention a number of pitfalls. On one hand, the calculation of ecological profile of each ASV needs to estimate their optimum (the taxa optimum with respect to an environmental gradient) and tolerance (a measure of niche-breadth) values according to the weighted average method proposed by ter Braak & Verdonschot (1995). The estimation of optimum and tolerance of each ASV needs to have access to large environmental datasets, which is not trivial. On the other hand, the spatial coverage (or geographical origin) of samples supplying the metabarcoding and environmental datasets can affect the calculation of ecological profiles of ASVs. Since ecological profiles of ASVs are calculated for a given environmental gradient, depending on the available sites, it is not advisable to reuse profiles obtained for a given region in other regions where environmental conditions may be different. Therefore, the taxonomy-free approach can provide important ecological information as long as the environmental characterization of samples is good. In other terms, the taxonomy-free approach suffers from geographical generalization until now.

Table 1
Advantages and pitfalls of different identification strategies.

	Beta diversity	Assignment success	Ecological information
ASV	+++	N/A	+
Species/genus	++	+	++
Functional groups	+	++	+++

With the species-based approach, ecological information is more or less accessible depending on the species under consideration. For example, a great quantity of information is available in literature on the autecology of a few species, that are easily identified under microscope and detected in abundance in our samples, such as *Asterionella formosa, Chrysochromulina* sp.*, Plagioselmis nannoplanctica, Plankothrix bourrellyi*. This trend is not generalized, since in most cases the amount of ecological information available is relatively low for most species. The first reason is that the morphology of many species is difficult to differentiate. For example, this lack of morphological differentiation is observed in *Chlorella*-like taxa, such as *Mychonastes homosphaera* (Chlorophyceae), which have been regularly recorded in our samples. Although this species has been the subject of genomic (Liu et al., 2020), biomass production (Saadaoui et al., 2020), and growth (Malinsky-Rushansky et al., 2002) studies, little information is available on its autecology. A similar example is *Monomastix* sp. (Mamiellophyceae), rarely identified *in natura* with microscopy (Cambra & Hindák, 1998) and for which we therefore have no robust knowledge of its autecology. The second reason is that, although picoalgae are a dominant group in lake phytoplankton (Callieri, 2008); individuals from this group are difficult to detect under microscopy (Li et al., 2019). Therefore, traditional information on the autecology of picoalgae species is scarce and may be doubtful. One example is *Cyanobium rubescens,* the most abundant taxon in our samples, which displays a high diversity of genotypes (82 different ASV). *Cyanobium rubescens* is described as epiphytic on planktonic *Planktothrix rubescens* (Komárek & Anagnostidis, 1999), abundant in deep lake layers (Padisák et al., 1997; Selmeczy et al., 2015). However, given the ubiquity of this species in our samples and its high genetic diversity, our knowledge of its autecology needs updating. For instance, a metabarcoding study has shown that the important cryptic genetic diversity of picoalgae in lakes is strongly controlled by environmental parameters (Schallenberg et al., 2021), which had never been revealed before. The third reason is related to taxonomical changes and species division, which can blur autecological knowledge. This is the case of *Dangeardinia pseudopertusa*, a species often detected in our samples. This species comes from a revision of *Chlamydomonas* genus (Nakada et al., 2016), but no autecological information is associated with it.

The trait-based approach is widely used in ecology, particularly for phytoplankton, thanks to its ability to work on a manageable number of well-defined traits, and creates a more direct link with ecosystem functioning (Gagic et al., 2015; Litchman & Klausmeier, 2008). In addition, trait dynamics are based on physiological and biological processes (e.g., nutrient fixing and presence of heterocytes). Therefore, trait dynamics are well known and easily predictable along environmental gradients compared to species-based approach (Tapolczai et al., 2017). Another advantage is that traits are independent of ecoregions (Soininen et al., 2016), making the approach easily generalizable. In this study, functional group compositions were clearly different between the mesotrophic lakes (Geneva and Bourget) and oligotrophic lakes (Annecy and Aiguebelette). Moreover, for each lake, there are clear seasonal patterns of RFGs (Frossard et al., 2022; Jacquet et al., 2022; Rimet et al., 2022). To finish, the dynamics of these traits are easy to communicate to stakeholders who are interested in a straightforward lake phytoplankton functioning and quality assessment.

5. Conclusion

In order to successfully achieve the good ecological status of freshwaters set by the WFD, it is inevitable to establish a sufficiently reliable and robust toolbox for the proper ecological status assessment. The modern DNA sequencing-based methodology offers us a highly sensitive and efficient tool for analysing large numbers of samples within short time, and we need to find the optimal way to take advantage of this technology. The assignment of traits to sequences could provide a robust and ecologically founded method to assess the structure of phytoplankton communities and provides new elements to support the assessment of the ecological status of water bodies. Our study has shown that, depending on the objectives set, different approaches are available for sequence assignment, i.e. the taxonomic approach, the taxonomy-free approach and the trait-based approach. There is not one single optimal approach, as none of them simultaneously achieves high discrimination between samples, high sequence assignment success and robust, easily accessible ecological information. While ASVs are the most relevant for describing beta diversity, trait-based assignment is of real interest in terms of ecological information. In comparison with the two other methods, the trait-based approach can be a suitable tool to acquire deeper ecological information by partly overcoming the problematics of the incomplete reference libraries at species level. However, it is important to bear in mind that the variable level of phylogenetic conservatism between traits influences our ability to correctly assign sequences, with increased assignment reliability positively related to phylogenetic conservatism. Therefore, if the aim is to provide easily interpretable ecological information, from DNA metabarcoding data, we recommend opting for direct trait assignment rather than taxonomic assignment, which implies a loss of data. Combined with higher assignment success, trait-based approach offers a new method for assessing the ecological status of water bodies based on phytoplankton. This approach also opens new fields of application, including its testing on other biological compartments used as BQEs under the WFD for assessing the ecological status of water bodies.

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CRediT authorship contribution statement

Kálmán Tapolczai: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Frédéric Rimet:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Miloš Ćirić:** Writing – review & editing, Data curation. **Andreas Ballot:** Writing – review & editing, Data curation. **Christophe Laplace-Treyture:** Writing – review & editing, Data curation. **Benjamin Alric:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2025.113361.

Data availability

Data will be made available on request.

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