

# Changes in Ciliate Communities Reveal Modification of Lake Functioning Over the Last Century

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## Research Article

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# Abstract

Ciliates are unicellular heterotrophic organisms that play a key role in the planktonic and benthic food webs of lakes, and represent a great potential as bioindicator. In this study, we used the top-bottom paleolimnological approach to compare the recent and past (i.e. prior to major anthropogenic impacts) ciliate communities of 48 lakes located along an elevation gradient using metabarcoding techniques applied on sedimentary DNA (sed-DNA). Our results show an overall decline in the  $\beta$ -diversity in recent time, especially in lowland lakes which are more strongly exposed to local human pressure. Analyses of the functional groups indicate important restructuring of the trophic food web and changes that are consistent with several well documented environmental changes such as the widespread increase in deep water anoxia, changes in thermal stability and nutrient cycling. Our study demonstrates the potential offered by sed-DNA to uncover information about past ciliate communities on a wide variety of lakes and the potential of using ciliates as valuable indicators, integrating information from the pelagic to the benthic profundal (and littoral) zones. Through trait-based functional community approach, the ciliates provide additional valuable information on ecosystem functioning, thus offering more a holistic view on the long-term changes of aquatic ecosystems.

## Introduction

Lakes are largely recognized as integrators and sentinels of environmental changes<sup>1</sup>. Pressures from anthropogenic activities have largely increased in magnitude since the mid-twentieth century, a period that has also been referred to as the Great Acceleration<sup>2,3</sup>. More specifically, climate change and the human alteration of the landscapes can have a profound impact on the physical and chemical characteristics of lakes<sup>4,5</sup>, thereby influencing the communities living in and depending on these ecosystems<sup>6</sup>. While challenging, assessing the biological response to environmental changes over large geographical scales can provide important insight into the vulnerability of lakes to anthropogenic and climate forcing. The top–bottom paleolimnological approach allows such assessment for aquatic communities through the comparison of sedimentary archives of recent (i.e. top samples) to past communities (i.e. bottom sample)<sup>7</sup>. This comparative approach is quite powerful as it can be applied on large geographical scales and provides important insight into reference conditions (i.e. prior to major anthropogenic influences), thereby allowing an assessment of the amplitude of change<sup>8,9</sup>.

Although the diagenesis and archiving of DNA in the sediments through time is often discussed in literature<sup>10</sup>, previous studies have demonstrated that the genetic information of the micro-organisms living in the water column archives in the sediments<sup>11–13</sup>. The development of molecular biology techniques to target DNA molecules preserved in lake sediments (sed-DNA) largely expanded the field of paleolimnology over the last decade<sup>14</sup>. For instance, applying molecular biology tools in paleolimnological investigations allowed to reconstruct historical freshwater biodiversity<sup>15</sup>, as well as uncovered past ecological changes of a wide range of organisms, including specimens for which visible remains are not preserved<sup>16,17</sup>. This includes the reconstruction of the long-term dynamic of micro-

organisms<sup>18</sup> which represent the largest source of biodiversity and ecological functions, but have long been omitted from debates concerning the response of the global biodiversity, in part due to the lack of data<sup>19</sup>. As such, sed-DNA offers new opportunities to reconstruct a more holistic view of the long-term response of lakes to environmental changes<sup>18,20</sup>. For instance, through the implementation of new paleo-indicators, sed-DNA can allow to reconstruct the long-term dynamic of overlooked communities such as the communities of the microbial loop that play a key role in the functional ecology of lakes.

Ciliates are unicellular microorganisms occupying diverse ecological niches and a wide biogeographic range<sup>21</sup>. These protists display a large functional diversity, acting as predators of bacteria and other protists, including algae, as well as small metazoans<sup>22</sup>, while mixotrophic ciliates can significantly enhance primary production<sup>23,24</sup>. Altogether, they play a key role in aquatic pelagic and benthic food webs, especially in the transfer of energy and nutrient cycling from the microbial loop to the metazooplankton<sup>21,25</sup>. Long-term reconstruction of the microbial eukaryote diversity of large peri-alpine lakes reported a strong relative contribution of the ciliate communities to the total abundance of microeukaryotic communities and demonstrated that ciliates are sensitive to changes in phosphorus concentration<sup>13</sup>. Although the ciliates are good indicators of environmental changes<sup>25,26</sup>, they are seldom used in routine limnological surveys. Indeed, the analysis of ciliate communities through microscopic-based approach and morphospecies identifications is challenging<sup>27</sup>, mostly because the monophyletic group of ciliates includes very different organisms that display diverse and complex life cycles<sup>28</sup>. Recent advances in molecular biology and the development of more robust taxonomic libraries continue to strengthen eDNA and sed-DNA techniques<sup>28,29</sup>, thereby allowing to include these understudied groups and useful indicators for a more holistic ecological diagnosis.

In the present study, we combine metabarcoding methods and the top-bottom paleolimnological approach to reconstruct recent and past ciliate communities of 48 temperate lakes (Fig. 1), for which drastic changes in the composition of the micro-eukaryotic communities were previously observed as a response to the Great Acceleration<sup>18</sup>, with a significant increase in phototrophic and mixotrophic communities consistent with the global enhancement of primary productivity. Focusing on these same lakes, we aimed at exploring how the heterotrophic and mixotrophic group of ciliates responded to these recent changes by (1) assessing the amplitude of changes in the ciliate communities across a wide variety of lakes and (2) evaluating the potential of using the ciliate as indicators of functional and biological changes.

## Results

**Ciliates metabarcoding analyses.** The sequencing resulted in a total number of 2,746,319 DNA reads with an average of 28,650 reads per sample. After the filtering steps, 1,745,532 reads were retained and clustered into 2446 OTUs. Detailed information about the effect of bioinformatics treatments on the DNA reads and a summary of the resulting total number of OTU and number of reads taxonomically assigned

and associated to a functional trait are accessible in the supplementary information (Supplemental Tables S1, S2).

**Ciliates community diversity.** The NMDS based on the Bray-Curtis distance showed that the overall dispersion of the recent and past samples differed, with the distance between the samples and their geometric median being much more variable and greater for the bottom samples (Fig. 2).

The average distance between the samples and their centroid was significantly different between the past 0.79 and recent samples 0.35 (Wilcoxon test,  $p < 0.01$ ). However, there was no large displacement of the centroid relative to each other as illustrated by the overlapping confidence ellipses of the two groups (Fig. 2a). This pattern was even more pronounced on the NMDS calculated from the OTU table (Supplemental Fig. S1). The hierarchical analysis identified four clusters according to their community composition, separating most past samples from the bottom samples (Fig. 3).

More specifically, the clusters 1, 2 and 3 were only composed of past samples except for the cluster 2 which contained the present and past samples of two lakes that did not differ. The samples of cluster 1 were dominated by the Oligohymenophorea (Class) Scuticociliatia (Subclass), samples of cluster 2 by Colpodea (mainly Cyrtolophosidida), while samples of cluster 3 were dominated by Spirotrichea Hypotrichia (Fig. 3). The fourth cluster regrouped the samples for which the ciliate communities were dominated by the Class Litostomatea, subclass Haptoria. Although the fourth cluster regrouped past and recent samples, sub-clusters indicated a clear separation of the recent from the past samples. The SIMPER analysis applied on the subclass level indicated that the increase of the Haptoria, the Oligotrichia and Armophorea contributed by 26%, 11% and 2% respectively to the Bray-Curtis dissimilarity between the recent and past samples, while the decline of Colpodea, Hypotrichia, Scuticociliatia and Prostomatea contributed by 19%, 18%, 6% and 3% to the dissimilarity. The differential abundance analysis (DESeq2) provided information about the intensity of the changes between the recent and past samples<sup>30</sup> and applied at the Genus level indicated a significant increase of five genus and families of the subclass Haptoria, two genus and families of the subclass Oligotrichia and the genus *Metopus* of the class Armophorea, as well as a significant increase of the Peritrichia and Suctoria (Supplemental Fig. S2). The DESeq2 analysis also indicated a significant decline of *Urotricha* a genus of the class Prostomatea, two Scuticociliatia including *Cyclidium*, five genus and families associated to the class Colpodea, and five genus and families associated to the class Hypotrichia, as well as the significant decline of *Peniculia*. The regression tree analysis identified a significant split in the dataset at an elevation of 1400m, with a Bray-Curtis dissimilarity coefficient averaging 0.37 for lakes situated above 1400m in elevation while lakes situated under 1400m in elevation displayed an average of 0.57 (Fig. 4).

**Ciliates functional traits and lakes ecosystem functioning.** The DESeq2 indicated a significant increase of the facultative or strict anaerobe benthic ciliates while the benthic ciliates that require oxygen significantly declined in the recent period (Fig. 5).

This increase in facultative or anaerobe benthic ciliates was mostly influenced by the significant increase in the abundance of the genus *Metopus* (order: Armophorida, class: Armophorea) (Supplemental Fig. S2). Moreover, there was a significant increase in sessile ciliates (i.e. attached to a substrate), as well as a significant increase in the pelagic ciliates that tends to be found in the epilimnion or distributed in the entire water column during mixing periods<sup>31,32</sup>. In contrast, ciliates associated with the hypolimnion of the pelagic zone significantly declined. In addition, the same analysis applied on the foraging trait categories indicated a significant increase in mixotrophs and non-selective filters feeders (which includes some detritivores), while bacterivores declined significantly in recent samples (Fig. 5).

## Discussion

Over the last decades, lakes have been exposed to environmental changes (anthropogenic stressors and natural changes) with important implications on the biological communities. Recent paleolimnological studies have provided new insights on the long-term responses of microbial assemblages by using DNA-based methods<sup>15</sup>. Although previous paleolimnological investigations of overall microeukaryotes communities reported a strong response of the ciliates to nutrients inputs<sup>13,33</sup>, specific long-term changes in the ciliates communities structure and functional ecology had not yet been investigated. The present study represents the first paleolimnological reconstruction of ciliate communities and demonstrate the potential of using heterotrophic protists as indicators of environmental change at the community and functional levels.

Analyses of the ciliate communities indicate an overall decline in the  $\beta$ -diversity in recent times following the same trends as the overall micro-eukaryotes diversity<sup>18</sup>. Through the use of primers specifically targeting ciliates, our study brings an innovative perspective and provides additional information by highlighting changes in some heterotrophic and mixotrophic communities that were not previously revealed in the analysis of the overall microeukaryote communities using generalist primers. Interestingly, the ciliates did not undergo a large turnover but rather our results indicate a spatial homogenization of the diversity with a reorganisation of the community structure (i.e. switch in dominance) (Figs. 2 and 3). This spatial homogenization of the ciliate communities was marked by the replacement of three clusters by one homogeneous community dominated by *Haptoria* across all lakes. Biotic homogenization of communities is a well documented phenomenon that has been observed in terrestrial and aquatic ecosystems largely influenced by a reduction of environmental heterogeneity and the availability of diverse ecological niches<sup>34-36</sup>. In aquatic ecosystems, change in climate and productivity, and anthropogenic alteration of the watershed are the most prevalent causes of biotic homogenization<sup>37</sup>. Several of our studied lakes are exposed to similar stressors which includes nutrient enrichment, agricultural and urban development of watershed and climate change<sup>38-41</sup>. As such, our results support that these factors acted as deterministic filters selecting for a more homogeneous group of species dominated by generalist ciliates that display more flexible life strategies.

A stronger restructuring of the ciliate communities was observed in the low elevation lakes demonstrating that environmental changes in lowland lakes impacted several trophic levels, including non-photosynthetic protist communities. The geographical variation in the amplitude of changes in diversity and community turnover of microorganisms associated with an elevation gradient have been previously demonstrated in terrestrial<sup>42</sup> and aquatic ecosystems<sup>43</sup>. These patterns are explained by the more pronounced human footprint in lowlands<sup>42,44</sup>, including nutrient-enrichment in freshwater ecosystems<sup>6</sup>. Supporting this trend, the present day trophic status of our studied lakes was significantly higher for lowland lakes than for high elevation lakes (Supplemental Fig. S3). As such, human-induced nutrient increase might have influenced the observed changes in the ciliate community diversity of lowland lakes.

Changes in the dominant functional groups of ciliates in the 48 studied lakes highlighted modifications of the physico-chemical conditions and biotic interactions of the pelagic and benthic zones.

The recent increase in the ciliates that can display mixotrophic life strategies whereby ciliates harbor algal endosymbionts or sequester plastid from their prey<sup>45</sup>, suggests that mixotrophic ciliates are directly or indirectly responding to new environmental conditions<sup>45</sup>. Interestingly, mixotrophic organisms tends to benefit from a more flexible nutrition strategy and have been found to prone during transition phases between autotrophy-dominated and heterotrophy-dominated systems<sup>46,47</sup>. As such, they can easily adapt to the more frequent exposure to extreme events or highly-variable environmental conditions that have occurred in lakes over the last decades<sup>48</sup>. Moreover, *Limnostrombidium* (synonym of *Strombidium*, from marine waters) is a common freshwater genus regrouping specialists mixotrophic species that preferentially feed and use the chloroplast of picophytoplankton<sup>31,45,49</sup>. Their significant increase in recent time thus indicates that recent changes in autotrophic picoplankton dynamic and structure, that have been previously recorded in some of our studied lakes<sup>16</sup>, might have provided them with a competitive advantage. Importantly, however, more empirical studies are still needed in order to solidify inference made between the relative increase in mixotrophic life strategies and associated ecological conditions.

Mixotrophic ciliates play a major role in foodweb structure of freshwater lakes<sup>24,50</sup>. The mixotrophic ciliate can contribute to the enhancement of the primary production<sup>51</sup>, while as prey, they represent a more direct transfer of the solar energy to the zooplankton<sup>24</sup>. Through this process, mixotrophic ciliates can enhance the efficiency of carbon transfer and energy flow in the food web<sup>24</sup>. Their recent increase thus suggests that lakes might have undergone important trophodynamic changes as mixotrophy is becoming an increasingly important pathway in aquatic food webs. These results also highlight the importance of integrating the mixotrophic component in food web modelling and carbon flow studies<sup>52</sup>.

Change in the benthic ciliate communities indicates that the benthic environment has also been impacted by recent environmental changes. The significant increase in the facultative or obligate anaerobic ciliate associated with the benthos, such as *Metopus*, suggests that the ciliate communities have been directly

influenced by the widespread deoxygenation of temperate lakes<sup>5</sup> (Fig. 5 and Supplemental Fig. S2). The significant decline in the benthic and hypolimnetic ciliates associated with well-oxygenated conditions further support that the habitability of the sediment-water interface has been declining for this particular group of ciliates. The depletion of oxygen concentrations in the profundal zone of freshwater lakes is a well recorded global phenomenon that can have a pervasive impact on the overall ecosystem functioning<sup>53</sup>. These changes have been associated with stronger and longer thermal stratification, as well as a loss of water clarity, in part due to the increases in pelagic production<sup>5</sup>. Supporting this hypothesis, some of our studied lakes, where the strict anaerobe bacteriophage *Metopus* have been recently thriving, have experienced unprecedented episodes of eutrophication or cyanobacterial bloom over the last decades and subsequent periods of deep water hypoxia<sup>54–56</sup>.

Significant changes in several other functional groups of ciliate provide additional evidence of recent changes in aquatic food web structures and habitats. For instance, the significant increase in pelagic ciliates that preferentially lives in the stratified epilimnium further support that lakes have been exposed to longer and stronger periods of stratification in recent time. Furthermore, periphytic species, such as the sessile or sedentary forms Peritrichia and Suctorina, is consistent with more frequent pelagic blooms or macrophytes under higher nutrient and warmer conditions<sup>57,58</sup>. However, the lack of knowledge about the ciliate ecology, their biotic interactions and regulatory factors limit our capacity to decipher the relative importance of each potential stressors and the underlying mechanisms remain elusive.

Altogether, the diagnosis of the changes in the ciliate communities across the 48 studied lakes supports the use of ciliates as indicators of environmental changes<sup>59</sup> and provides evidence that the ciliate communities are strongly responding to the environmental changes that have occurred over the last century which includes widespread deoxygenation of deep waters, changes in thermal stratification and nutrient-enrichment. Playing a key role in the metabolic pathways of aquatic ecosystems<sup>51,60</sup>, they can provide valuable insight into the functional ecology of lakes, and their strong response recorded in the sedimentary archives suggests important changes in the main pathways for the transfer of energy within the microbial food webs<sup>61</sup>. Although, molecular studies of protist communities and working with ancient DNA is quite challenging with several aspects that need to be taken into consideration for future studies (as summarized in Methods section of the Supplemental Material), the present study as well as several previous studies<sup>27,51,62</sup> have shown the great success of using such approaches to assess environmental changes in aquatic ecosystems. Their integration to environmental assessment using high-throughput sequencing and metabarcoding technologies is thus promising, providing a more holistic overview of the response of aquatic ecosystems to environmental changes. This is even more relevant as the science is moving toward ecosystem-wide food web modelling<sup>20,51,63</sup>, and protists, as key players of the microbial food-web, serve an important function of recycling carbon and energy in lakes.

## Methods

### Study sites, sediment core collection and subsampling

A total of 48 lakes were used in this study (Fig. 1, Supplemental Table S3). The studied lakes were selected to be located along a large elevation gradient and displayed various physico-chemical characteristics (Supplemental Table S3). For all 48 lakes, the sediment cores were retrieved between 2010 and 2016 (cf. Keck et al.<sup>18</sup> for more detailed information on each lake) from the deepest part of the basin using a UWITEC gravity corer. Prior to subsampling, the core was air protected by a double layer of plastic wrap and stored in the dark at 4°C.

A top-bottom technique was used to provide a simplified assessment of the amplitude of change in the ciliate community diversity and composition. Briefly, a top sample corresponding to recent time and a bottom sample corresponding to the past were isolated from the cores. Importantly, the top samples (i.e. the last decade) were selected to be a few centimeters down the core to avoid bias associated with active benthic ciliate taxa and early diagenesis processes<sup>12</sup>. The depth of the bottom samples was chosen for each core in order to correspond to the pre-“Great Acceleration” period<sup>2,3</sup> (i.e. nineteenth century) which was determined using a combination of several approaches (i.e. X-ray fluorescence, radiocarbon and radionuclides <sup>210</sup>Pb and <sup>137</sup>Cs; cf. the supplementary material and methods in Keck et al.<sup>18</sup> for more detailed information on each lake). In order to ensure that each sample covered at least 10-15 years, the thickness of the sediment samples was individually adjusted. Sub-sampling for DNA analysis was conducted in a clean and controlled environment using strict laboratory protocols to avoid contamination by modern DNA<sup>15,18</sup>.

## Molecular Analysis

Two DNA extractions were performed on 0.5 g of wet sediment for each sample using the NucleoSpin® soil kit, according to the manufacturer instructions (Macherey-Nagel, Düren, Germany). The same DNA extracts as in Keck et al.<sup>18</sup> were used. Refer to the Method section of the Supplementary Material for more details regarding the laboratory protocol and conditions applied for the DNA extraction. A nested-PCR targeting the V7 region of the 18S rRNA gene was used to do the inventory of the ciliate community. In the first step, a set of primers was used to target a specific DNA region for ciliates of 800bp CS322F (5'-GATGGTAGTGTATTGGAC-3') and 1147R (5'-GACGGTATCTRATCGTCTTT-3')<sup>64,65</sup>. The first PCR was performed in a total volume of 25 µL containing 1 µL of DNA extract, 2.5 µL of 10X NH<sub>4</sub> reaction buffer, 2 µL of 50mM of MgCl<sub>2</sub>, 0.5 µL of 100mM dNTP, 1.25 µL of each primer at 10 pmol/µL, 2 µL of 10mg/mL BSA and 0.1 µL of 5Ci BioTaq (Bioline). The amplification cycle included an initial denaturation at 95°C for 10 minutes followed by 20 cycles of 15 seconds at 94°C, 15 seconds at 57°C and 30 seconds at 72°C. The amplicons were then subjected to a final 10 minutes extension at 72°C. The second PCR was then applied on the products of the first PCR using the general eukaryotic primers 960 F (5'-GGCTTAATTTGACTCAACRCG-3') and NSR1438 (5'-GGGCATCACAGACCTGTTAT-3'), previously used by Keck et al.<sup>18</sup>, amplifying in DNA fragment of about 250bp. Molecular tails were added to the forward primer (5'-CTTTCCCTACACGACGCTCTTCCGATCT-3') and to the the reverse primer (5'-GGAGTTCAGACGTGTGCTCTTCCGATCT-3'). The second PCR was performed in a total volume of 25 µL containing 0.8 µL of DNA from the first PCR, 2 µL of 10X NH<sub>4</sub> reaction buffer, 1.6 µL of 50mM of MgCl<sub>2</sub>, 0.4 µL of 100mM dNTP, 1 µL of each primer with molecular tails at 10 pmol/µL, 1.6 µL of 10mg/mL BSA

and 0.06 µL of 5Ci BioTaq (Bioline). The amplification cycle included an initial denaturation at 95°C for 2 minutes followed by 20 cycles of 30 seconds at 94°C, 30 seconds at 57°C and 45 seconds at 72°C. The amplicons were then subjected to a final 10 min extension at 72°C. The nested-PCR protocol was applied on each DNA extractions separately, the full volume of the final products resulting from the two DNA extracts of the same sample were then pooled and sent to GeT-PlaGe (Plateforme Génomique 31326 CASTANET-TOLOSAN Cedex) for amplicon purification, library preparation and paired-end (2 × 250 bp) sequencing on a MiSeq Illumina instrument (San Diego, CA, USA).

The reads were demultiplexed and R1/R2 reads assembled into contigs by the sequencing platform who provided one fastq files per sample. The high-throughput sequencing data were then cleaned in Mothur 1.45.1<sup>66</sup>. Filtering steps were used to conserve DNA sequences of 350±50 bp in length, with no ambiguities (N=0), 10 or less homopolymer (max homopolymer=10) and no mismatch was allowed in the primer sequence. The data was dereplicated in order to work with Individual Sequence Unit (ISU). ISUs were then aligned using an aligned version of the Silva 18S database restrained to the V7 region and ISUs that were not fully aligned to the Silva 18S barcode were removed. The detection and removal of chimera was done using Vsearch as implemented in Mothur with default parameters. The taxonomic assignment of the ISU was done using a curated version of the Protist Ribosomal Reference database PR2<sup>67</sup> “pr2\_version-4.12.0\_18S\_cil\_cur” (available on Zenodo repository system: <https://doi.org/10.5281/zenodo.5163167>) and using the command `classify.seqs()` and the method wang with a confidence score threshold of 75% and 100 iterations. Following this first taxonomic assignment, the ISU represented with only one read or that were identified as “unknown” or “Eukaryota\_unclassified” were removed. The ISUs were then clustered into molecular Operational Taxonomic Unit (OTU) using the furthest neighbour algorithm with a similarity threshold of 97%. Finally, the command `classify.otu()` was used to taxonomically assign the OTUs based on the first taxonomic assignment of the ISUs with a confidence threshold of 80%. The OTUs that did not belong to the Phylum Ciliophora were removed. The taxonomic affiliations were checked and harmonized manually using the classification from Gao et al.<sup>68</sup>. In order to study changes in the functional groups, the ecological preferences (i.e. preferred limnetic habitat or foraging traits; cf. Table 1) were indexed for the OTUs for which the taxonomic affiliation was fine enough (at least assigned to the family rank); otherwise, the category “Unknown” was given. The association of OTUs to their functional traits was done through an exhaustive literature review, the foraging traits categories created were inspired from a combination of several previously published categories based on the feeding ecology of ciliates<sup>69,59,70,22</sup>. Table used with the information about the functional traits is available on open access (<http://doi.org/10.5281/zenodo.5534333>).

Table 1  
Description of the ciliates functional traits categories

Categories associated with the limnetic habitat	Benthic (oxic conditions)	Ciliates that can in found in the littoral zone or well-oxygenated bottoms.
	Commensals or Parasites	Freshwater ciliates that are either endocommensals of bivalves or ectocommensals of fish, or parasites of fish.
	Facultative or obligate anaerobe	Facultative or obligate anaerobe living in the benthic environment but also includes some taxa able to live in anaerobic deep waters.
	Pelagic	Ciliates that are found in high abundance in the epilimnion during the stratification period and can be found in low abundance throughout the water column during spring and fall mixing.
	Pelagic (hypolimnion)	Ciliates that preferentially lives in the hypolimnion or metalimnion during the stratification periods and are generally found in low abundance during mixing periods. Ciliates from this group do not withstand anoxic conditions.
	Sessile	Ciliates for which their life cycle includes a stage attached to a substrate. Usually stalked ciliates.
Categories associated with foraging strategies	Algivores	Herbivorous ciliates
	Bacterivores	Ciliates that exclusively feed on bacteria, these ciliates are usually associated with the benthic environment or found in the metalimnion of highly productive lakes
	Carnivorous	Ciliates that feeds on other ciliates or even small metazoans
	Commensals and parasites	Regroup parasites, bacterivores and histophage ciliates. Commensals and parasitic ciliates were kept separated as they are more likely to be directly influenced by the presence/absence of their hosts rather than influenced by changes in the biotic and abiotic factors of the surrounding environment.
	Filter-feeders (non-selectives)	Passive filter-feeders and detritivores. Ciliates from this category are associated with highly productive aquatic environment or environment with a high concentration of dissolved organic matter (i.e. often found in polluted waters or waste water treatment plants).
	Fungivorous	This categories contains only one species found in our samples <i>Pseudoplatyophrya nana</i>
	Mixotrophs	Phagotrophic ciliates that harbor algal endosymbionts or sequester plastid from their algal prey. Mixotrophic ciliates thus tends to be also algivores.
	Predators of small protists	Omnivorous ciliates feeding on algae and other small ciliates

# Statistical analysis

Analyses were done using the R software version 3.11<sup>71</sup> using the *vegan* package<sup>72</sup>, the *rpart* package<sup>73</sup> and DESeq2 package<sup>30</sup>.

The normality and homogeneity of variance of the environmental variables were tested using a Shapiro-Wilk test of normality<sup>74</sup> and a Fligner-Killeen's test<sup>75</sup>, respectively. If the variables were not normally distributed even after transformation non-parametric test were used. A Spearman correlation analysis was applied to study the relationship between the environmental variables. To assess the relationship between lake topology and trophic status the Kruskal test was used; whenever the Kruskal test<sup>76</sup> was significant, to assess which categories of trophic status were significantly different, a Wilcoxon rank sum test as Post Hoc test was applied using the False Discovery Rate approach by Benjamini and Hochberg<sup>77</sup> to adjust the p value for multiple testing (Supplemental Fig. S3).

In order to harmonise data and allow comparison between samples, reads were transformed into relative abundance. Changes in the  $\beta$ -diversity of the ciliates at the community level between the past and recent samples were investigated using a Bray-Curtis dissimilarity matrix that was built based on the relative abundance data at the subclass level. Results were visualized on a NMDS (Non-metric Multidimensional Scaling analysis). To evaluate the change in dispersion and thus in diversity between the past and recent samples, the distances between the samples and the geometric median for each group ("recent" and "past") were calculated. The difference between the median of each group was then tested using the Wilcoxon rank sum test in order to evaluate the overall displacement of the recent and past samples. A hierarchical cluster analysis on Bray-Curtis distances using unweighted pair group method with arithmetic mean (UPGMA) was used to identify if a clear separation could be observed between and within recent and past samples. A SIMPER analysis (SIMilarity PERcentage)<sup>78</sup> was performed on the relative abundance data to calculate the contribution of each subclass to the overall Bray-Curtis dissimilarity between the recent and past samples. The most abundant species can have a high contribution even when they do not differ among groups, as they tend to display the highest variance<sup>72</sup>, as such, the proportion in SIMPER contribution, in average change between recent and past samples, as well as the total number of reads were compared to each other.

In order to evaluate the relative importance of known physical characteristic of the lakes (i.e. continuous variables: elevation, maximum depth, surface area of the lake, surface area of the watershed) to the amplitude of changes in the  $\beta$ -diversity, a univariate regression tree analysis was applied on the Bray-Curtis dissimilarity between the recent and past sample of each lake. For categorical data (i.e. Trophic Status), an analysis of variance (ANOVA) was used to compare the Bray-Curtis dissimilarity coefficients between categories.

The difference in abundance between the recent and past samples was also evaluated for each functional group and at the Genus level using the DESeq2 framework applied on the raw count data<sup>30</sup>.

The results were expressed as “log2foldchange” and provided an indication of the intensity of the changes between the recent and past samples.

For the creation of the map, the software QGIS<sup>79</sup> was used, for all other figures, we used the package *ggplot2*<sup>80</sup> and the color palette from the package *scico*<sup>81</sup> to create scientifically derived color-maps accessible for people with colour-vision deficiencies<sup>82</sup>.

## Declarations

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### Authors contributions

I.D., L.M., D.G., D.R. contributed to the conception and general design of the work; I.D. developed the designed the eDNA study and obtained financial support for the study, provided advice on data analysis and interpretation; B.C. performed the data analysis, interpretation of the data and wrote the first draft of the manuscript; V.V. and F.K conducted the bioinformatics treatments of the molecular data and contributed to the data analysis. All authors contributed to the writing of the MS and gave final approval for publication.

### Competing interests

The authors declare no competing interests

### Additional information

Raw metabarcoding data are available at <https://doi.org/10.5281/zenodo.5163167>

Taxonomical and functional trait affiliation used are available at <http://doi.org/10.5281/zenodo.5534333>

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## Figures

Figure 1

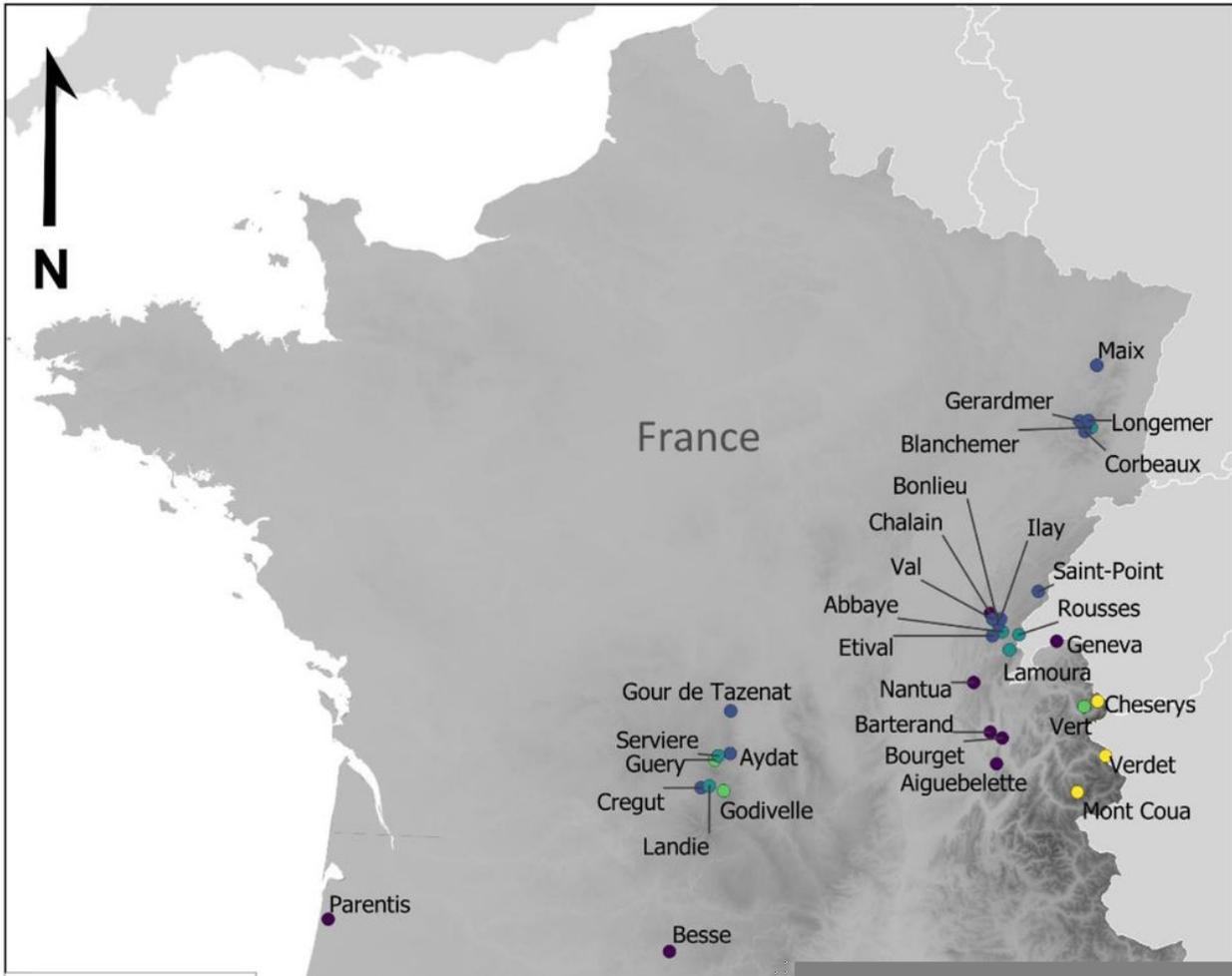


Figure 1

Location of the 48 studied lakes. The colours correspond to associated elevation range (cf. legend), the grey gradient illustrates the elevation terrain (DEM; source: <https://srtm.csi.cgiar.org/>) within the geopolitical borders of France.

Figure 2

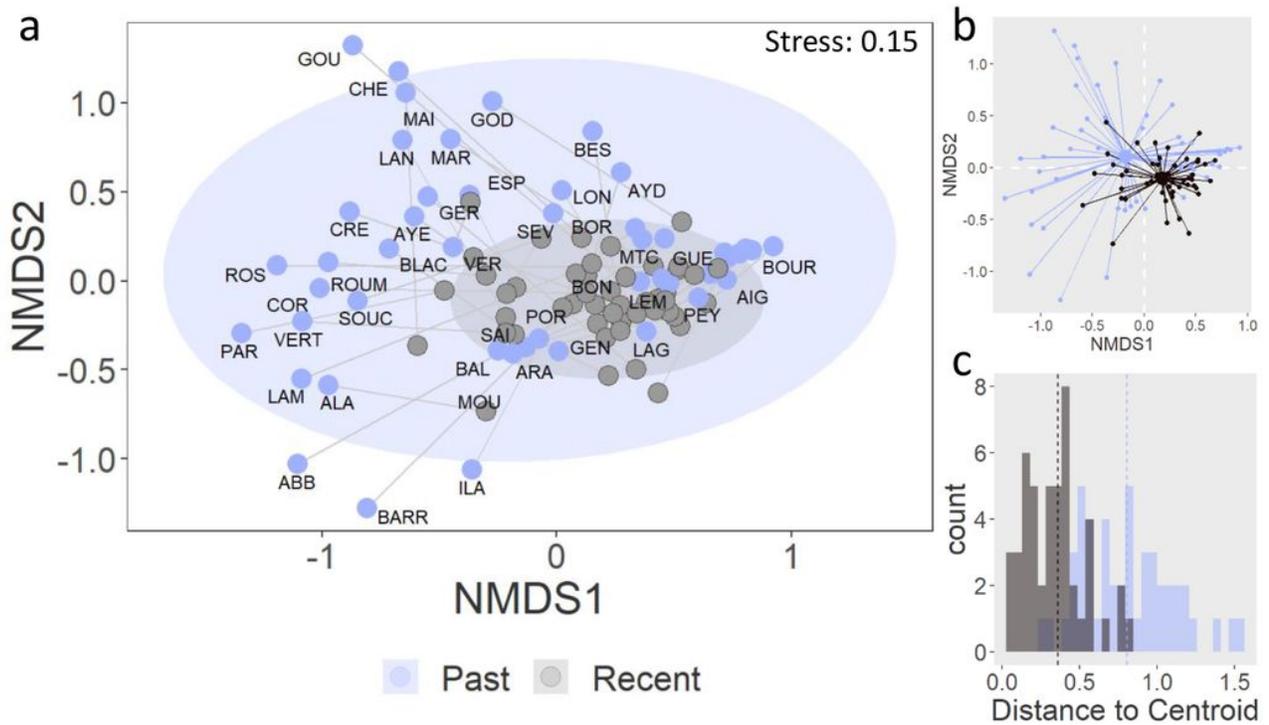


Figure 2

(a) NMDS of community compositions of the recent (grey dots) and past (purple dots) samples with 95% confidence ellipses represented for each group. Only the past samples are labelled with their corresponding lake code (cf. Table S3), the grey lines connect recent and past samples from the same lake. Note: some labels are missing to avoid overlapping labels. (b) Spider plot showing the location of the geometric median of the recent and past samples group and illustrating the distance of the recent and past samples related to the geometric median of their respective group. (c) Distribution of the distances between samples and group geometric median for recent and past samples, the geometric median of each group is indicated by the dotted line.

Figure 3

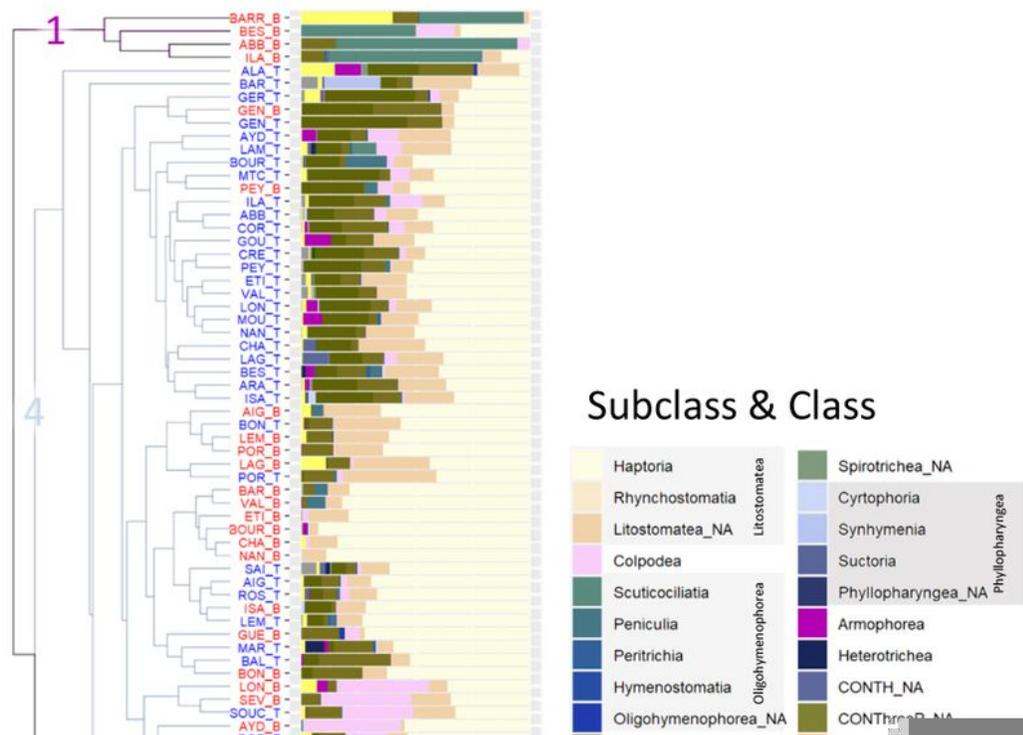
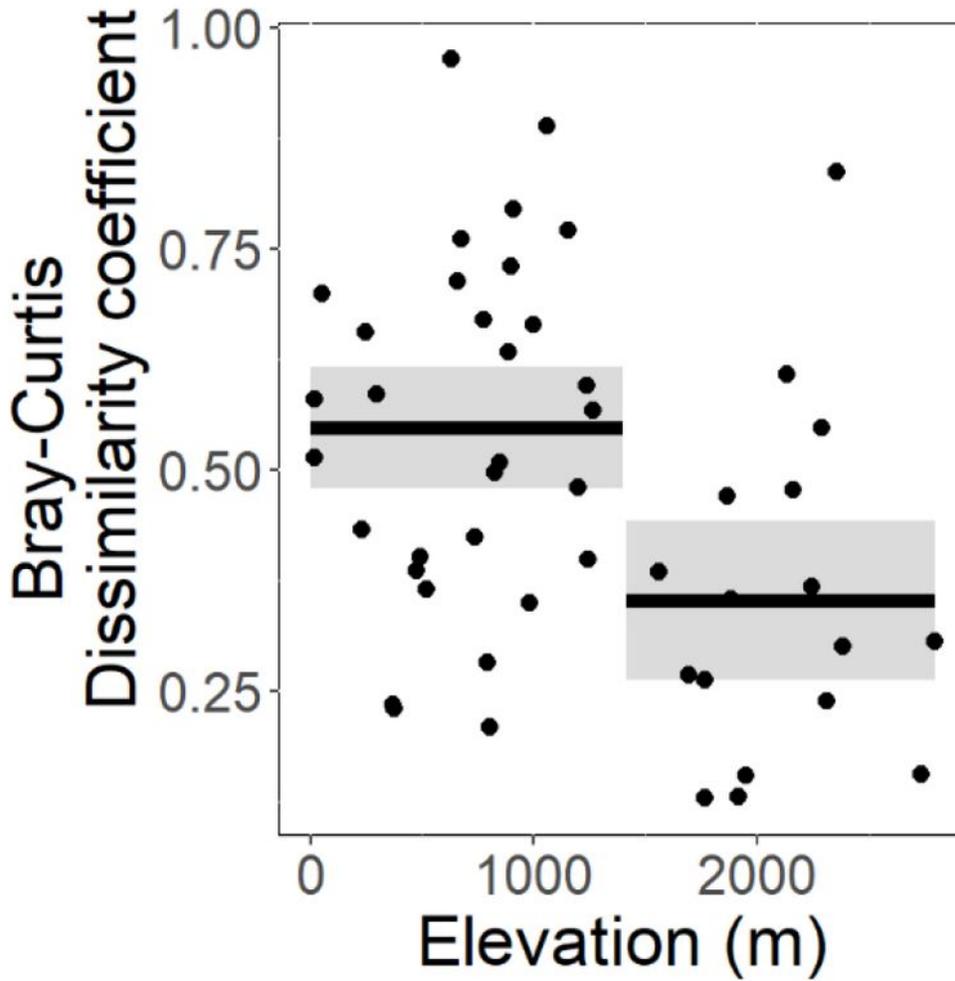


Figure 3

Dendrogram illustrating the results from the hierarchical cluster analysis. The ciliate community composition the dominant Order or Class levels (i.e. Class\_NA, if the Order level could not be assigned) of each sample is illustrated on the right side of the dendrogram and expressed as relative abundance (%). Each sample is named by their corresponding lake code (cf. Table S3) followed by the code “\_T” or “\_B” indicating the recent (i.e. “recent”, in blue) or past (i.e. “bottom”, in red) samples respectively.

# Figure 4



**Figure 4**

Distribution of the Bray-Curtis dissimilarity coefficients between recent and past samples (black dot) relative to lake elevation (m). Fitted regression tree model (n =48 lakes) identified a split at 1400m and is represented by black lines (mean values). Gray shading represents the 95% confidence intervals around means.

Figure 5

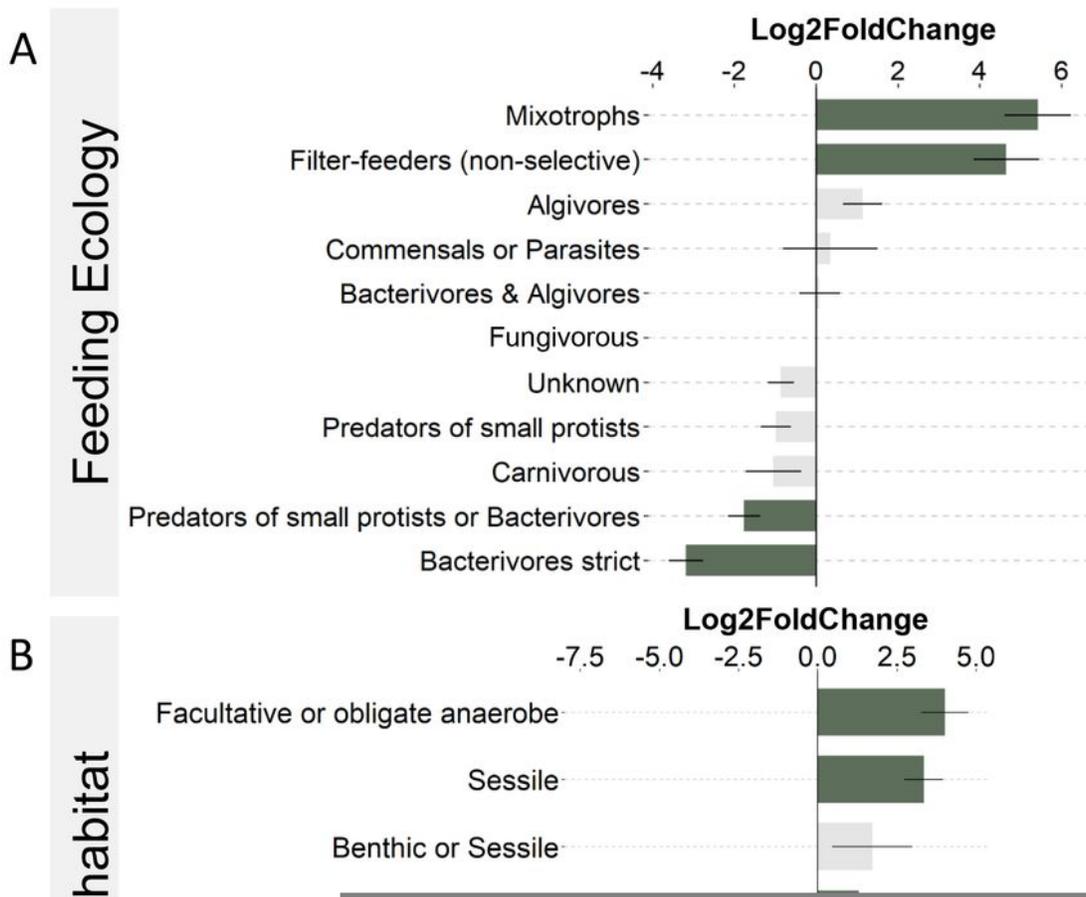


Figure 5

Amplitude of change in the functional groups between the recent and past samples according to the feeding ecology (A) and limnetic habitat preferences (B). Magnitude of change is expressed in log2 fold change, as estimated by the DESeq2 analysis (n= 48 lakes). Dark green bars represent groups for which the change was found significant according to the two-sided Wald test corrected with the Benjamini and Hochberg method (p-value < 0.05). Horizontal lines show the standard error. Note: OTUs that were

associated to several foraging traits (e.i. Bacterivores and Algivores, Predators of small protists or Bacterivores) were either OTUs for which the taxonomy was not fine enough to select only one type of foraging traits or OTUs which corresponded to ciliates that displayed very diverse feeding strategies.

## Supplementary Files

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