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RESEARCH ARTICLE

Nonlinearities in phytoplankton groups across temperate high mountain lakes

Teresa Buchaca[1] | Jordi Catalan[2,3]

[1]Integrative Freshwater Ecology (CEAB-CSIC), Blanes, Spain

[2]CREAF, Cerdanyola del Vallès, Spain

[3]CSIC, Bellaterra, Spain

Correspondence
Teresa Buchaca
Email: buch@ceab.csic.es

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Abstract

1. High mountain lakes are increasingly recognized as sentinel ecosystems of global change. Monitoring phytoplankton changes or reconstructing their composition from sedimentary records can help identify systemic changes in these lakes and their catchments.
2. This study aimed to evaluate the distribution of the major phytoplankton groups in high mountain lakes across environmental gradients and identify tipping points in relative dominance. The phytoplankton groups were estimated using pigment-based chemotaxonomy in 79 lakes in the Pyrenees selected to cover the bedrock and elevation gradients. Fifty-four environment variables were considered, including in-lake and catchment descriptors.
3. Redundancy analyses showed that in-lake descriptors override the explicative capacity of landscape variables. Generalized additive models and multivariate regression trees showed that water hardness, trophic state and food-web descriptors were, in this order, the most influential factors determining phytoplankton group dominance. Calcium concentration of about 200 $\mu eq\,L^{-1}$ defined the threshold between soft waters—with chrysophytes and chlorophytes showing a higher affinity for them—and harder waters that favour diatoms and cyanobacteria. Across the trophic gradient, there was a threshold at ~5 $\mu g\,L^{-1}$ of total phosphorus (TP), chrysophytes being dominant below that TP value and cryptophytes above. The dominance of chlorophytes and cryptophytes increased with the density of macrozooplankton. Chrysophytes were significantly lower and diatoms higher in lakes with fish.
4. *Synthesis*. The relative abundance of phytoplankton groups in temperate high mountain lakes responds in a nonlinear way to the hardness of the water in the range 20–1195 $Ca^{2+}\,\mu eq\,L^{-1}$ and the trophic state in the range 0.94–19 $TP\,\mu g\,L^{-1}$. The thresholds across water hardness and trophic state gradients coincide with studies based on other organisms, pointing to a robust typology for mountain lakes that should be considered when selecting global change sentinel lakes and anticipating abrupt transitions across these thresholds.

KEYWORDS
abiotic factors, biotic factors, chemotaxonomy, high mountain lakes, marker pigments, phytoplankton, Pyrenees

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1 | INTRODUCTION

High mountain lakes are sentinel ecosystems of the current global change (Catalan et al., 2013; Moser et al., 2019) and witnesses of the climate and environmental past (Catalan et al., 2014; Pla & Catalan, 2005; Schmidt et al., 2004). A suite of indicators (proxies) is used to monitor the ongoing change and decipher the information recorded in the sediments. Phytoplankton is particularly suitable for lake trophic state assessment (Reynolds et al., 2000). In temperate lakes, all algal groups increase in biomass with total phosphorus (TP) with different degrees of curvilinearity in the relationship across the wide range of nutrient levels (Jeppesen et al., 2005; Watson et al., 1997). This wide nutrient range has been used to develop trophic indexes in which chrysophytes are usually located at the oligotrophic extreme and cyanobacteria at the more productive (Phillips et al., 2013). However, considerable variation in phytoplankton composition can be found when relatively short segments of the wide TP range are considered, particularly within the less studied oligotrophic range in which high mountain lakes are located. This high variability indicates that other factors could be relevant, such as other nutrients and stoichiometric relationships among them (e.g. nitrogen (N)), main ions, dissolved organic carbon (DOC) and grazing pressure, among others. Although phytoplankton studies have a long tradition in mountain lakes (Rodhe et al., 1966; Tilzer, 1973), assessing the environmental drivers across a broad range of lakes has been more limited (Tolotti et al., 2006). Establishing appropriate reference benchmarks of expected variation in high mountain lakes becomes essential for sentinel site selection, trend interpretation and regional upscaling of those changes that could anticipate major ecological shifts (Cook et al., 2023). This study aimed to determine the variation of the major phytoplankton groups across the wide environmental gradients in high mountain lakes. We looked at spatial patterns to infer responses to those gradients and unveiled tipping points of compositional change. In this study, nonlinearity is defined as when equal-step incremental changes in an environmental variable produce minor effects on phytoplankton composition over a range, but the same size of change produces a large effect over another range. The transition between the two ranges constitutes a tipping point, or threshold, if abrupt.

We considered two main types of environmental variables for investigating the response of phytoplankton groups: (i) those variables defining the proximal environment of the phytoplankton cells at which their physiology responds, which may have a fast influence and (ii) those variables at higher spatial and temporal scales that may condition and define the limits of fluctuation in the proximal environment and the covariance across space, which may have a diffuse but persistent influence on the phytoplankton communities (Levin, 2000). In the particular case of high mountain lake districts, elevation, bedrock nature, vegetation, the hydrological network and lake morphology, among other landscape features, downscales and influence the proximal physical and chemical conditions and resource availability (Catalan, Curtis, et al., 2009) that phytoplankton can experience added to biotic constraints such as predation and parasite interactions (Lürling, 2021). Due to the microbial nature of phytoplankton and the potential fast turnover of the communities, we hypothesized that the explicative power of proximal variables (e.g. nutrients or grazing) would outperform variables at higher integrative levels (e.g. altitude, catchment vegetation or lake size) for explaining the instantaneous phytoplankton composition.

Furthermore, provided that most lakes are within highly oligotrophic conditions, we hypothesized that gradients other than those of nutrients might show a significant influence on the composition of phytoplankton groups and their coexistence (Li & Chesson, 2016), more specifically, those related to the main water chemistry, conditioning, for instance, dissolved inorganic carbon availability and exchanges across phytoplankton cell membranes, and those related to predation, or, more widely, food-web structure. Finally, we expected that changes in the relative composition of the main phytoplankton groups would show curvilinear relationships with the gradients, and consequently, tipping points would be identifiable, which will constitute the basis for anticipating significant changes in phytoplankton communities through space or time. Nutrient enrichment experiments in specific sites have shown that these nonlinear responses occur (Zufiaurre et al., 2021), and more generally, discontinuities in dominance over large environmental gradients appear to be the result of niche conservatism of eco-evolutionary processes (Wiens et al., 2010).

To test these hypotheses, we initially considered numerous environmental variables, encompassing several categories: the geographic setting; catchment characteristics (e.g. bedrock and vegetation); the lake biotic environment (e.g. zooplankton, fish and macrophytes); the physical environment (e.g. temperature and light); and water chemistry (e.g. nutrients, main ions and DOC). We performed partial redundancy analyses within each category to reduce the number of variables and select those with the most significant non-redundant explicative capacity. With all the selected variables, we performed another redundancy analysis that confronted all the categories and tested whether large-scale or proximal variables showed the highest explicative potential. The selected variables were used to explore the nonlinearities in the phytoplankton group distribution and the tipping points using nonlinear trend fitting, generalized additive models and regression trees.

Because we implemented a procedure extremely severe against collinearity effects, the selected variables were expected to show low shared explicative capacity. Nevertheless, some of them could be understood as representative of sets of variables that, in nature, tend to occur in a highly correlated way. For instance, a trivial case is all those variables related to water hardness. Alkalinity, pH, calcium, magnesium and ionic strength, among others, appear highly correlated in freshwaters, so in a demanding selection of variables, only one will likely be selected, although they may be involved in different aspects of algal cell physiology. Therefore, the selected variables and associated sets will be considered when discussing the identified environmental gradients.

High mountain lakes are one of the most comparable continental ecosystems globally, particularly across the temperate zones

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because of their headwaters' nature and similar origin related to glacial erosion in most cases (Moser et al., 2019). Nevertheless, regional and subregional differences occur according to predominant local factors (e.g. bedrock nature) and atmospheric forcing (e.g. N atmospheric contamination) (Burpee et al., 2022). The high mountain lakes of the Pyrenees were considered in our study. Several thousands of lakes and ponds across the massif encompass a wide variation in elevation, catchment vegetation and bedrock. This diversity allowed a survey design likely including a relevant part of the world's chemical and physical variation across temperate mountains. However, the Pyrenees have received high atmospheric nitrogen deposition since the 1960s, which should have substantially changed the N:P in waters since pre-industrial times. Currently, there is an extended P limitation that perhaps could be reverting because of the recent increase in P deposition (Camarero & Catalan, 2012). In any case, the study was unlikely to identify patterns related to N limitation. Photosynthetic active radiation (PAR) and ultraviolet radiation (UVR) are also factors that potentially could influence phytoplankton composition (Sommaruga, 2001). They were not considered because of logistic limitations. Indeed, their influence was minimized by sampling at depths of approximately iso-irradiance in each lake.

2 | MATERIALS AND METHODS

2.1 | Study sites and environmental variation

The study was based on a survey of 82 lakes from mid-July to the end of August 2000 across the entire range of the Pyrenees (Figure 1; Table S1). During this period, summer stratification occurs in most lakes (Catalan et al., 2002), and phytoplankton communities can be assumed to be in a similar successional stage. The lakes were selected to cover the main bedrock, elevation and geographical variation within the range. Only one or, exceptionally, a few lakes were sampled in cirque basins that included many close lakes to reduce potential spatial covariance because of the proximity and also to facilitate covering the entire massif with the available resources. The environmental variables considered (54) were grouped into five categories (Table 1) to evaluate the partial and hierarchical influence on the phytoplankton groups. Water chemistry (15 variables) included nutrients, major ions and DOC. Water samples for chemistry were collected near the lake outlet. Water samples were analysed for pH (Orion Research model 231 pH-meter, at laboratory temperature, 20°C), conductivity (PTI-10 conductimeter) and alkalinity (Gran acidimetric titration). Major anions (sulphate, nitrate and chloride) and major cations (calcium, magnesium, sodium and potassium) were analysed by capillary electrophoresis (CIA-4000 Waters Capillary Ion analyser). Ammonium was determined by the phenol hypochlorite method (Solorzano, 1969), and nitrite was determined by the sulphanilamide and n-naphthyletilenediamide method (Grasshoff & Almgreen, 1976). Total nitrogen was determined by persulfate digestion (Grasshoff & Almgreen, 1976), followed by UV spectrophotometry. Particulate phosphorus, total phosphorus and total dissolved phosphorus were oxidized to phosphate by acid-persulfate digestion (Grasshoff & Almgreen, 1976), and soluble reactive phosphorus was concentrated using chromatographic cartridges. Phosphate was analysed by the malachite green method (Camarero, 1994).

The physical environment (12) was characterized by considering morphological, thermal, light and littoral substrate characteristics. During the survey, temperature profiles, Secchi disk depth, maximum depth and littoral substratum composition were measured in the field. The biotic environment (8) included macrophyte and fish presence (assessed using ancillary information and visual inspection during the survey), the organic content of the top sediment (loss on ignition, LOI) (Dean, 1974) and planktonic components. Rotifers and macrozooplankton (MZP) were collected with vertical hauls using plankton nets of 40 and 200 μm mesh size, respectively. Bacterial biomass was determined following Straškrabová et al. (1999).

The description of the catchment (16) included the catchment area and geological characteristics determined using cartographic information (Spanish and French Geological Maps) and GIS techniques (Casals-Carrasco et al., 2009), estimates of precipitation and duration of the ice cover inferred by extrapolation methods

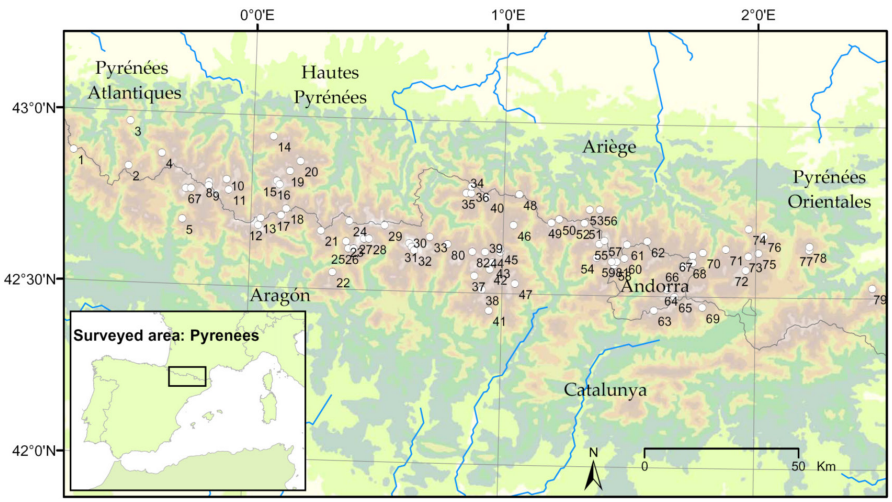


FIGURE 1 Location of the lakes surveyed in the Pyrenees. See Table S1 for the lake name and georeferences.

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TABLE 1 Environmental factors considered in the study.

Variable	Code	Maximum	Minimum	Median
Water chemistry				
pH	pH	8.96	4.53	6.98
Conductivity (μS cm^{-1}, 20°C)	Cond	191	5.2	22
Ammonium (μeq L^{-1})	NH4	3.9	0.26	1.2
Calcium (μeq L^{-1})	Ca	1195	20	127
Magnesium (μeq L^{-1})	Mg	557	4	14
Sodium (μeq L^{-1})	Na	76	0.3	20
Potassium (μeq L^{-1})	K	22	0.0	3
Alkalinity (μeq L^{-1})	Alk	1696	<0	123
Sulphate (μeq L^{-1})	SO4	1240	10	41
Nitrate (μeq L^{-1})	NO3	18	0.02	4.7
Chloride (μeq L^{-1})	Cl	37	3	6.8
Total nitrogen (μg L^{-1})	TN	968	44	162
Total phosphorus (μg L^{-1})	TP	19	0.94	3.3
Silica (mg L^{-1})	Si	5.0	0.02	0.67
Dissolved organic carbon (mg L^{-1})	DOC	3.7	0.01	0.79
Physical environment				
Lake area (ha)	L_area	44.65	0.63	5.69
Maximum lake depth (m)	Depth	100	2.1	16.5
Rocky littoral substrate (%)	Lit_Rock	100	0	68
Sandy littoral substrate (%)	Lit_Sand	80	0	20
Organic littoral substrate (%)	Lit_Org	100	0	5
Water residence time (years)	Resid	4.46	0.01	0.25
Ice-cover duration (days)	Ice Cover	229	114	184
Surface water temperature class (categoric)	Temp	16	0	12
Thermocline presence (binary)	Termo	1	0	1
Temperature gradient presence(binary)	Grad	1	0	0
Complete mixing presence (binary)	Mix	1	0	0
Maximum lake depth irradiance (%)	Io (Zmax)	65.7	0	6.1
Biotic environment				
Top sediment organic matter content (%)	LOItop	38.6	2.6	18.5
Chl-*a* (μg L^{-1})	ChlConc	19.1	<0.01	1.18
Bacterial and filamentous biomass (μg C L^{-1})	Bacteria	93.3	1.74	16.8
Fish presence (binary)	Fish2	1	0	1
Macrozooplankton density (ind m^{-3})	MZP	95,646	5	4532
Total zooplankton (ind m^{-3})	Zooplankton	1,029,605	76	7690
Rotifers (ind m^{-3})	Rotis	945,169	12	1564
Macrophyte species presence (binary)	*Potamogeton* spp. (Pot sp.), *Sparganium angustifolium* (Sparg. Ang), *Ranunculus* spp. (Ran sp.), *Subularia aquatica* (Sub aq.), *Isoetes* (Iso sp.), *Nitella* (Nit sp.), *Myriophyllum alterniflorum* (Myrio altern.)			
Catchment descriptors				
Catchment area (ha)	Carea	5438	13	115
Headwater lake (binary)	Headwater	1	0	1
Catchment orientation (eight binary variables)	N, S, E, W, NE, NW, SE, SW			
Precipitation (mm $year^{-1}$)	Precip	1904	1221	1603
Metamorphic rocks (%)	GeolMet	100	0	0
Plutonic rocks (%)	GeolPlut	100	0	60

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TABLE 1 (Continued)

Variable	Code	Maximum	Minimum	Median
Detrital rocks (%)	GeolDet	100	0	0
Carbonate rocks (%)	GeolCarb	90	0	0
Glacial deposits (%)	GeolGlac	5	0	0
Glaciers (%)	Glaciers	15	0	0
Bare rock (%)	Bare	100	10	55
Moorland (%)	Moorland	10	0	0
Meadows (%)	Meadow	90	0	30
Shrubs (%)	Shrubs	60	0	0
Coniferous woodland (%)	Con_wood	35	0	0
Deciduous woodland (%)	Dec_wood	5	0	0
Geographic setting				
Latitude N (degrees)	Lat	42.97	42.45	42.65
Longitude E (degrees)	Lon	2.21	−0.71	0.95
Altitude (m a.s.l.)	Alt	2880	1620	2300

(Thompson et al., 2005, 2009), and vegetation categories evaluated during the survey. Finally, the geographic setting (3) was defined by coordinates and altitude. Permits for sampling were obtained for all lakes from responsible entities, including Parc National des Pyrénées, Parc Naturel Régional des Pyrénées Ariégeoises, Parc Naturel Régional des Pyrénées Catalanes, Parque Nacional de Ordesa y Monte Perdido, Parque Natural de Posets-Maladeta, Parc Nacional d'Aigüestortes i Estany de Sant Maurici, Generalitat de Catalunya, Govern d'Andorra, among other regional and local authorities.

2.2 | Phytoplankton sampling and chemotaxonomy

Pigments were used for quantifying the relative dominance of high-rank taxonomic phytoplankton groups in 79 lakes. The relationship between chlorophyll and phytoplankton biomass may be highly influenced by light conditions (Felip & Catalan, 2000). To facilitate the lake comparison, we standardized pigment sampling by collecting water at an iso-irradiance depth in the deepest part of each lake at 1.5 times the Secchi disk depth. At this depth, between 1% and 10% subsurface irradiance penetrates in summer, usually coinciding with a typical deep chlorophyll maximum (DCM) (Callieri et al., 2007; Catalan et al., 2002). The DCM results from a phytoplankton growth optimum at a depth balancing nutrient and light availability (Pechlaner, 1971). In shallow lakes, with >10% surface irradiance reaching the bottom and weak stratification, DCM develops near the lake bottom, as early studies have already observed (Rodhe et al., 1966). Therefore, the sample was taken between 1 and 2 m above the surface sediment in 29 lakes where the Secchi depth reached the sediment surface.

Water samples were collected using a polyethylene tube connected to a flask and a manual vacuum pump. A volume between 1.5 and 2 L was filtered using Whatman® GF/F filters (Maidstone, UK), which were wrapped in aluminium foil and kept cold before freezing (−20°C) within 3–6 h.

The pigments were extracted from frozen filters using a probe sonicator (50 W, 2 min) with 90% acetone. The extract was filtered through Whatman Anodisc filters (0.1 μm) and analysed by HPLC. The HPLC system was equipped with a Waters 600E solvent delivery system, a Waters 717 autosampler set at 4°C, a C18 column (dimensions: 250 × 4.6 mm, particle size: 5 μm; Spherisorb-ODS1, Waters Corporation, Milford, US) and a Waters 996 photodiode array detector. The detector was set at 440 and 660 nm to integrate the carotenoid and phorbin peaks, respectively. The pigments were separated based on modifying the method described by Kraay et al. (1992). After injection of the sample (40 μL), pigments were eluted by a linear gradient from 100% solvent A (0.3 M ammonium acetate in methanol: acetonitrile: MilliQ water, 51:36:13 (v/v/v)) to 75% A and 25% B (ethyl acetate: acetonitrile, 70:30, (v/v)) for 5 min followed by 5 min and 20 min, respectively, of isocratic hold at 75% A and 100% solvent B. The flow rate was 1.2 mL min^{-1}. The solvent composition was returned to initial conditions on a 5-min gradient, followed by 5 min of system equilibration before injection of the following sample. Pigments were identified by comparison with a library of pigment spectra obtained from extracts of pure algae cultures from the Culture Collection of Algae and Protozoa (CCAP, Oban, Scotland, UK). Chl-*a*, Chl-*b* and β,β-carotene standards were obtained from Sigma Chemical Co. Ltd. (UK). The extinction coefficients used for calculations were obtained from the literature (Jeffrey et al., 1997; Rowan, 1989).

The contribution of each algal group to phytoplankton biomass was estimated in terms of Chl-*a* using CHEMTAX (Mackey et al., 1996; Schlüter et al., 2006). The method works by algorithmic iteration and requires a first estimate of the marker pigment to Chl-*a* molar ratios (initial ratio matrix; H_0) appropriate for the algal classes expected in the sample (Descy et al., 2009). The matrix of

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the pigment ratio is varied by a small amount in each iteration, and the class abundance is recalculated. The class sum is checked against the measured total Chl-*a*. A steepest descent algorithm estimates the final best-class abundances and pigment ratios (H_m). The process is repeated until the least-squares fit satisfies predetermined limits ($\varepsilon = 0.005$). CHEMTAX gives the best fit of contributions of the predefined taxa to total Chl-*a*. The advantage of this method is that it distinguishes between algal groups with qualitatively identical pigment compositions by differences in pigment ratios. We used between 1 and 4 marker pigments per group, which included chlorophytes, chrysophytes, cryptophytes, diatoms, dinoflagellates and cyanobacteria (Table S2). The initial values of pigment ratios (H_0) were obtained from the output matrix (H_m) of a previous study in a Pyrenean lake (Buchaca et al., 2005), except for diatoms and cyanobacteria that were obtained from the literature (Mackey et al., 1996; Soma et al., 1993) because these groups were not sufficiently abundant in that study. Specific pigment ratios to chlorophyll may change for different reasons (Goericke & Montoya, 1998). One possibility is exposure to different levels of irradiance. Therefore, we standardized the irradiance level during sampling according to the Secchi disk measurements. However, it was impossible for some shallow lakes, see above and light levels varied compared with the rest. Therefore, we checked whether it was better to consider a unique set of samples or split them into those sampled at 1.5 times the Secchi disk depth and those at higher irradiance. No significant differences were found (Figure S1); therefore, the reported results correspond to a unique set that includes all lakes. The comparison between the input (H_0) and output ratios (H_m) indicated minor changes except for two diatom pigments: diadinoxanthin was reduced by half, and diatoxanthin increased slightly (Table S2). Therefore, the H_m ratios reported in this study can be recommended as starting H_0 for studies in other mountain lake areas.

2.3 | Data analysis

The hierarchy and selection of factors that provided a more independent explanation of the distribution of the relative abundance of phytoplankton groups were assessed by partial redundancy analysis (RDA) (Ter Braak, 1994). Computations were performed using CANOCO (Ter Braak & Šmilauer, 2002). Bonferroni-adjusted forward selection was used to reduce redundancy between variables. The significance of the variable was tested with Monte Carlo permutation (999 permutations, $p < 0.005$) (Besag & Clifford, 1989). The variables of water chemistry were normalized by logarithmic transformation. Initially, we independently considered the five groups of environmental factors (RDA-1 to -5; Table 1; Table S3). Subsequently, we performed a final analysis (RDA-6), including the significant variables of each group, to achieve a minimum explicative model that underlined the main linear gradients of the segregation of the phytoplankton groups. The significant variables at this unified level were further investigated using techniques that consider nonlinear responses. Nevertheless, it should be considered that each of the variables finally selected may represent a complex of colinear variation because the minimum model procedure used was quite demanding to avoid false positives (i.e. Bonferroni adjustment and partial RDAs).

A model-free, locally weighted sequential smoothing technique (LOWESS) (Cleveland, 1979) was used to visualize the nonlinear relationships between the phytoplankton groups and the primary environmental variables selected in the redundancy analysis. Generalized additive models (Hastie & Tibshirani, 1991) were applied to evaluate the response of the relative abundance of phytoplankton groups to these environmental variables using the mgcv R package (Pedersen et al., 2019). Generalized cross-validation (GCV) was used as the smoothing selection method, except for cyanobacteria, where restricted maximum likelihood (REML) was applied to avoid apparent overfitting. Finally, the primary thresholds in the dominance of the phytoplankton groups were identified by a multivariate regression tree (MRT) (De'Ath, 2002). The MRT divides the response matrix of the composition of algal groups into clusters. A simple rule based on environmental thresholds defines each split. The splits are chosen to minimize the dissimilarity of sites within clusters. A tree represents the clusters and their dependence on the environmental data graphically. The number of clusters included in the tree was defined by the solution that minimized the cross-validated relative error (CVRE) (Borcard et al., 2011). The mvpart package of R version 3.1.1 (R Foundation for Statistical Computing) (Therneau et al., 2004) computes the partition and the cross-validation steps required to build an MRT.

3 | RESULTS

3.1 | Phytoplankton composition

Phytoplankton composition was inferred from CHEMTAX in 79 lakes. Chrysophytes were the dominant algal group in most lakes (43%), followed by cryptophytes (19%), chlorophytes (18%) and diatoms (15%), while cyanobacteria and dinoflagellates were dominant only in a reduced number of lakes (5% and 1%, respectively). Beyond absolute dominance, the proportion of chrysophytes was generally remarkable: >30% in about 50% of sites (Figure 2). Chlorophytes, diatoms and cryptophytes occasionally achieved high dominance but were below 25% of total Chl-*a* in ~75% of cases. The contribution of dinoflagellates reached 40%, but it was not detectable in about 80% of the lakes, while cyanobacteria reached 48% and were generally present in slightly lower proportions than chlorophytes, diatoms and cryptophytes (Figure 2).

3.2 | Environmental gradients

The partial RDA models ranked the explicative capacity of each set of variables as RDA-1 model water chemistry (17%, with Ca^{2+} and TP selected), RDA-2 model biotic environment (14.3%, fish and

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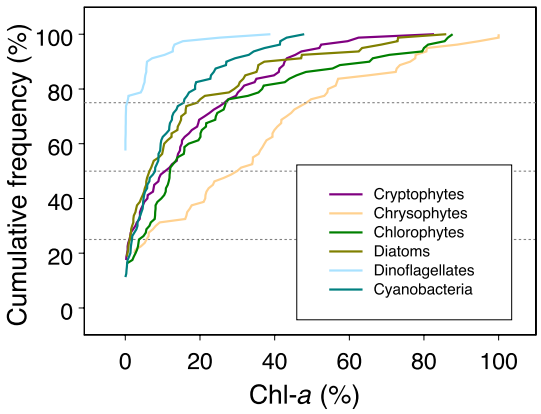


FIGURE 2 Likelihood of the relative contribution of each taxonomical group to total community biomass expressed as the cumulative percentage of the Chl-*a* (%) values ranked from low to high in the set of lakes. Reference horizontal lines indicate quartiles of the distribution. The more diagonal the curve is, the higher the group's dominance in general terms.

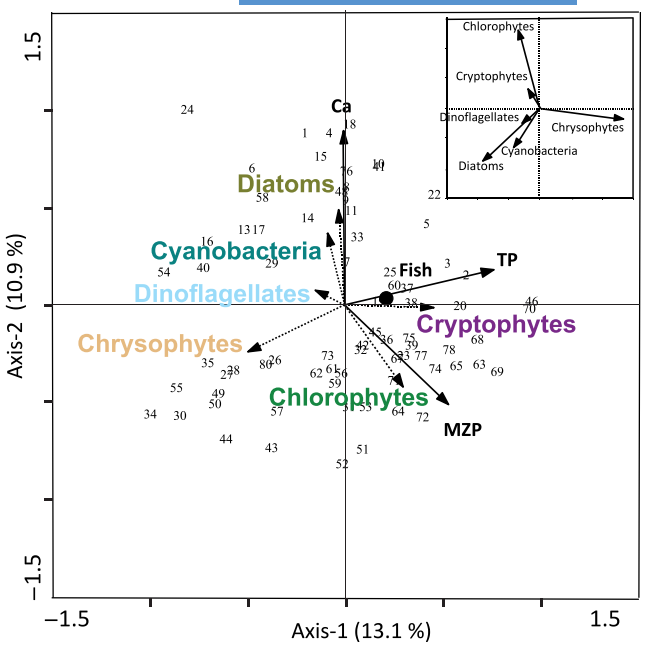


FIGURE 3 Ordination diagram (RDA-6) showing the linear combination of environmental variables that describe the main gradients of variation in the composition of the phytoplankton group. The inset shows a principal component analysis of the phytoplankton groups. In this ordination, the axes, calculated with environmental constraints, are similar to the RDA ones (with adequate rotations), which indicates that the environmental variables used did capture the principal axes of phytoplankton variation. The abbreviated names of the environmental variables and units are provided in Table 1. The Fish variable is plotted using the centroid value because of its binary character.

MZP), RDA-3 model geographic location (9.8%, Lon and Alt), RDA-4 model physical environment (6.7%, Temp) and RDA-5 model catchment descriptors (5.1%, GeolPlut). The shared variance between the selected variables within each model was generally low (Table S3), as expected from Bonferroni adjustments. When the eight selected variables were combined in a single model (RDA-6), only four remained after forward selection: Ca^{2+}, TP, fish presence and MZP, which explained 26.3% of the phytoplankton group variance, with low shared variance between the variables (Table S3). Those variables not selected corresponded to landscape descriptors (Lon, Alt, GeolPlut), indicating that their explicative value was embedded and overridden by the proximal environmental descriptors.

The two main axes of the integrative RDA-6 explained a similar amount of phytoplankton variation (Figure 3): axis-1 defined a trophic gradient, with a positive main load of TP but also of fish and MZP, and axis-2 indicated a water hardness gradient, with a positive main Ca^{2+} load and negative by MZP. Cryptophytes appeared to be closely associated with higher trophic states and chrysophytes with lower ones (axis 1). Chlorophytes were notably correlated with the MZP density. Cyanobacteria and diatoms were positively associated with the Ca^{2+} gradient.

3.3 | Phytoplankton distribution along environmental gradients

Despite wide scattering, the total Chl-*a* (TChl-*a*, nmolL^{-1}) tendencies along the primary environmental gradients were well defined (Figure 4, note the logarithmic scales in the axes). TChl-*a* increased with TP, particularly below 2.5 µgL^{-1}, and declined with Ca^{2+} levels. TChl-*a* >2 nmolL^{-1} (2.24 µgL^{-1}) were only found in lakes with Ca^{2+} <200 µeqL^{-1} and increased with the density of MZP until this reached ~10^4 ind m^{-3}. These patterns were roughly reproduced by the absolute biomass of most phytoplankton groups expressed as a contribution to total Chl-*a* (Chl-*a* (pmolL^{-1}); Figure 4). However, sharp differences appeared in relative proportions (Chl-*a* (%); Figure 4). The proportion of chrysophytes declined when the concentration of TP increased throughout the gradient, while the cryptophytes showed the opposite trend. A crossover between the absolute dominance of the two groups occurred at 6 µgL^{-1}. Chlorophytes were more relevant as TP concentration increased but declined above 10 µgL^{-1} TP concentration.

The absolute Chl-*a* decline at >200 µeqL^{-1} Ca^{2+} concentration was particularly marked for dinoflagellates (Chl-*a* (pmolL^{-1}); Figure 4). The chrysophyte decline started at lower values (~50 µeqL^{-1}) despite maintaining relative dominance along the whole gradient. Diatoms and cyanobacteria increased their relative dominance at higher Ca^{2+} levels. The latter became the co-dominant algal group with cryptophytes and chrysophytes (~25% each) in lakes with the highest Ca^{2+} concentrations.

Despite the parallel increase of total Chl-*a* and MZP density up to 1 nmolL^{-1} and 10,000 ind m^{-3}, respectively, the relative contribution of each phytoplankton group to this pattern changed strongly (Figure 4). The contribution of chrysophytes decreased with increasing MZP density. However, the group remained dominant until about the point of change, when Chl-*a* started to decline with an increase in MZP. At ~30,000 ind m^{-3}, cryptophytes and chlorophytes become

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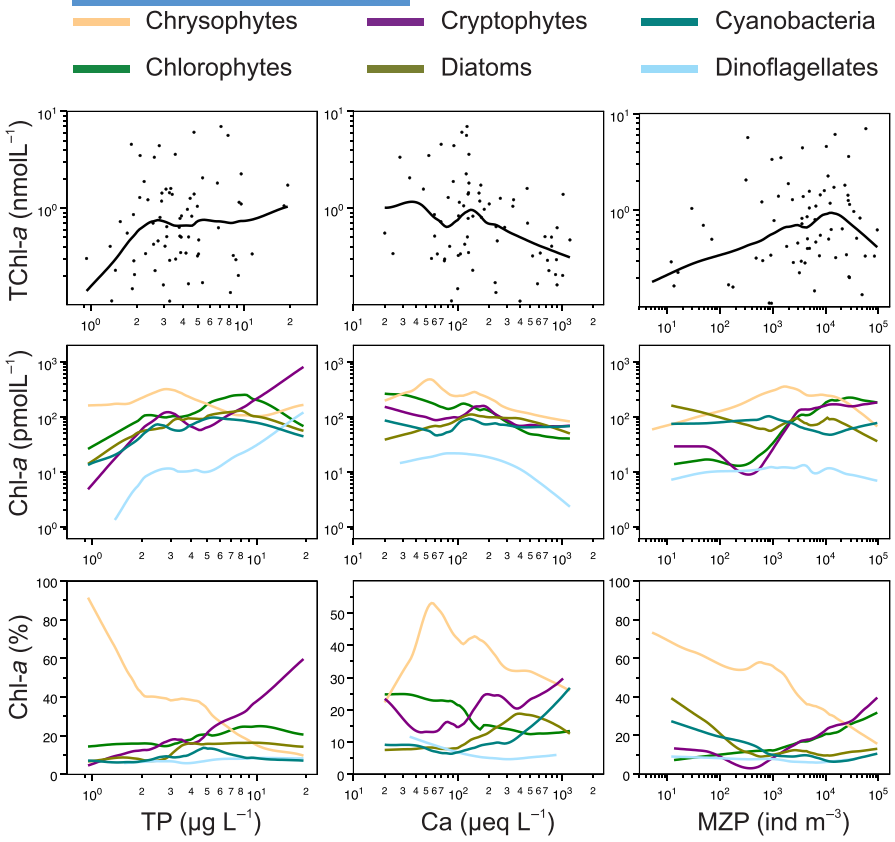


FIGURE 4 Total Chl-*a* (TChl-*a*; nmol L^{-1}) and changes in Chl-*a* of the taxonomic groups in absolute (Chl-*a*; pmol L^{-1}) and relative values (Chl-*a*; %) along the primary environmental gradients (TP, Ca^{2+} and MZP) fitted with LOWESS smoothing (span = 0.5). Note the logarithmic axes.

dominant. The absolute and relative nonlinear increase of these two groups started at much lower MZP concentrations (500–1000 ind m^{-3}). Diatoms, dinoflagellates and cyanobacteria were relatively insensitive to MZP concentrations in absolute biomass terms, although they showed a sharp relative decline when MZP increased at low densities (<1000 ind m^{-3}).

The scattering around the main observed tendencies was wide. Therefore, we performed GAMs to evaluate the significance of the relative abundance patterns found for each group, also considering the presence of fish (Figure 5; Table S4). The explained deviance ranked as chrysophytes (49%), cryptophytes (42), chlorophytes (32), diatoms (27), cyanobacteria (25) and dinoflagellates (2) (Table S4). TP was particularly significant for chrysophytes and cryptophytes, while Ca^{2+} concentration resulted significantly for cyanobacteria, diatoms and chlorophytes. MZP resulted significantly only for cryptophytes. Chrysophyte levels were lower and diatoms higher when fish were present. No significant factors for dinoflagellate dominance were identified.

The multivariate regression tree analysis evaluated the gradients in a common framework and determined the primary thresholds of the distribution of the phytoplankton groups in the study lakes (Figure 6). The regression tree analysis represented 26% of the variation, similar to RDA-6. The primary partition was related to Ca^{2+}, showing a threshold of ~200 µeq L^{-1}, above which diatoms and cyanobacteria were favoured and included about 1/3 of the study lakes. Within the softer water range, the following threshold was related to MZP density (~3000 ind m^{-3}), above which cryptophytes and chlorophytes increased relevance over chrysophytes, which were particularly characteristic of lakes in the low trophic state (TP < 5 µg L^{-1}). Dinoflagellates did not respond preferentially to any of these thresholds.

4 | DISCUSSION

Using high mountain lakes for global change surveillance requires considering the primary nonlinear aspects of the distribution of organisms. Environmental thresholds help define lake typologies that discern which lakes make sense to compare and which do not, and when within a tendency, a critical shift may occur. This study highlights three aspects. First, the biological distinction between soft and hard waters has to be considered. Our results add to previous studies and confirm that ~200 µeq L^{-1} alkalinity is a primary threshold for the distribution of autotrophs in freshwaters (Reynolds, 2006). Second, ultraoligotrophy occurs below ~5 µg TP L^{-1}, where chrysophytes dominate, at least in soft water conditions. These two thresholds would define the archetypic high-mountain temperate lakes of soft and ultraoligotrophic waters common in mountain catchments of low reactive bedrock and poorly developed soil. Third, more productive waters, still under oligotrophic conditions, also drive larger macrozooplankton densities (>3000 ind m^{-3}), favouring chlorophytes and cryptophytes. The dominance of these two latter groups indicates transitions to mesotrophic states. Cyanobacteria, which appear throughout the study lakes, decline at the more productive and soft water extremes. Finally, our study is inconclusive about fish effects since no trout-related trophic cascades were evident. Likely, the composition of phytoplankton is not the most sensitive indicator

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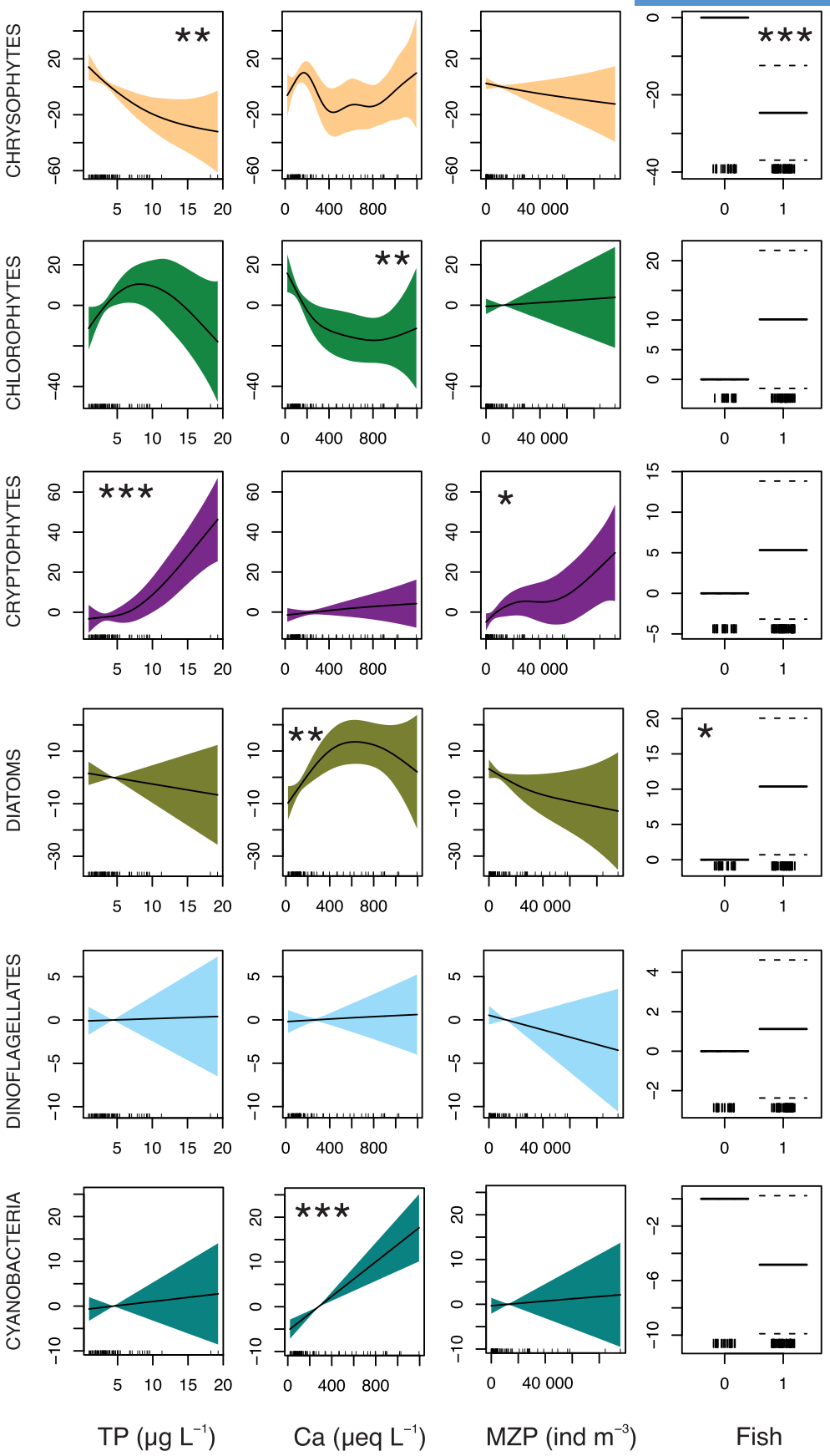


FIGURE 5 Generalized additive models of the relative abundance of phytoplankton groups. Colour silhouettes indicate 95% confidence intervals. Significant tendencies are indicated (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$). Different colours for each group are used to establish quick visual references with other figures.

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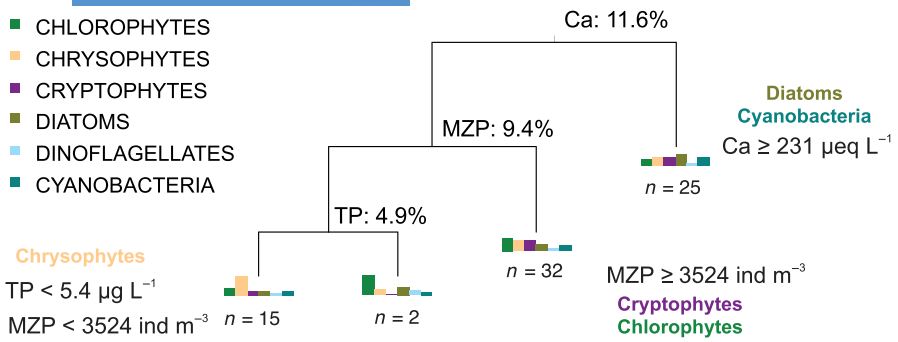


FIGURE 6 Multivariate regression tree for phytoplankton group composition data, showing the variance explained by each node in the tree and threshold values. Bar plots show the compositional mean at each node of the multivariate phytoplankton groups; the number of sites (*n*) is indicated.

of the possible impact of trout on mountain lakes. On the contrary, amphibians and invertebrates from the littoral zone are impacted by trout and, even more, by minnows when present (Miró et al., 2018; Osorio et al., 2022). Therefore, the presence of trout does not appear to be an obstacle that interferes with the contribution of phytoplankton to the global change sentinel role of high mountain lakes. The results indicate that proximal environmental variables are better indicators of the main changes in phytoplankton groups than large-scale variables. The amount of plutonic rocks in the catchments is the only variable with some explicative capacity in case of the lack of chemical data and can be used for large-scale planning.

4.1 | Chemotaxonomical phytoplankton composition

The composition of phytoplankton can be assessed by morphological microscopy inspection, amplicon or metagenomic molecular approaches, and chemotaxonomically. This study used pigments as markers of the composition of high-rank taxonomic phytoplankton. High mountain lakes often show low phytoplankton biomass and species diversity, with small cells that are difficult to identify by microscopy. Pigment-based methods offer a good alternative for quantifying the relative dominance of high-rank taxonomic groups (Buchaca et al., 2005; Jeffrey et al., 1997), facilitating surveys across many lakes and long-term time series. Likewise, pigments are the most persistent signature of the phytoplankton community in the sediment record.

Chrysophytes, cryptophytes and chlorophytes were the most abundant phytoplankton groups, while dinoflagellates and cyanobacteria had a lower abundance. Among these two latter groups, dinoflagellates were found in only a few lakes, while cyanobacteria were detected in most lakes. The general dominance of chrysophytes is a characteristic of high mountain lakes that were also found in a study including hundreds of sites in the Pyrenees, using DNA molecular taxonomy (Ortiz-Álvarez et al., 2018) and in studies of phytoplankton seasonality in single lakes, using microscope observations (Capblancq, 1972; Felip et al., 1999). In fact, chrysophyte richness and biomass dominance have been found in several temperate mountain lake districts (Straškrabová et al., 2009; Tolotti et al., 2003, 2006) in studies that also pointed to the relevance of cryptophytes and dinoflagellates. Beyond mountain lakes, chrysophytes appear as the distinct and dominant group in the freshwater oligotrophic extreme of pan-European datasets (Phillips et al., 2013). There is no reason to think this could not be a world-wide feature within soft water high mountain lakes in temperate climates.

Dinoflagellates have low pigment content concerning biovolume (Buchaca et al., 2005) and a higher preference for epilimnetic layers (Felip et al., 1999). These two circumstances could explain the low signal of pigments from this group found in this study that focused on deeper waters. In a seasonal study conducted in Lake Redon (Pyrenees), Buchaca et al. (2005) estimated 0.14 μg Chl-*a* L^{-1} attributable to dinoflagellates during the ice-free period. The comparison between biovolume measured using microscopy and pigments showed no pigment signal below 0.1 $mm^3 L^{-1}$. Furthermore, dinoflagellate pigment composition is not as coherent among species as in other groups (Rowan, 1989). The relevance of this group in our study have been likely underestimated.

Because mountain lakes are essentially oligotrophic, they could be thought free of planktonic cyanobacteria, as this group is commonly associated with extreme eutrophication in freshwater. However, early observations already showed that this is not the case (Capblancq, 1972), although they usually belong to small coccal forms (Negro et al., 2000) that might be confused with heterotrophic bacteria. The introduction of fluorescence microscopy helped recognize the wide distribution of this picoplankton (Callieri & Stockner, 2002). In our study, although the contribution of cyanobacteria was always low, the group marker pigments (zeaxanthin and myxoxanthophyll) were found in many lakes (72), confirming the overlooked influence of this group in ultraoligotrophic systems (Callieri, 2008).

4.2 | The biological distinction between soft and hard waters

Headwaters are usually associated with soft waters because the orogenic process usually facilitates the predominance of crystalline bedrocks in mountain ranges, resulting in lower rock weathering rates and, thus, waters with low ionic strength acid-neutralizing capacity (Psenner & Catalan, 1994). Ca^{2+} is usually the principal cation that provides alkalinity to waters, resulting in a strong correlation between Ca^{2+}, alkalinity, pH and conductivity in temperate mountain ranges (Camarero et al., 2009). Because the forward selection of variables in the RDAs performed removed highly

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correlated variables, only Ca^{2+} was kept as an explanatory variable of the phytoplankton group distribution, but, in fact, it represented a whole environmental package related to water hardness (sum of divalent cations) and the pH-carbon dioxide-bicarbonate systems. The GAM and MRT analyses showed that the Ca^{2+} threshold was the most determinant in the relative composition of the main phytoplankton groups. From a phytoplankton perspective, soft waters can be defined as those with <200 μeq Ca^{2+} L^{-1}. Above this value, diatoms and cyanobacteria increased relevance. Our results agree with research conducted by other authors that found diatom phytoplankton dominance in lakes with pH between 7.6 and 8.2 in the French Alps, so Ca^{2+} levels likely were above 200 μeq L^{-1} (Jacquemin et al., 2019). Similarly, lakes in the Canadian Rockies with pH > 8 show high fucoxanthin levels, likely indicative of diatom dominance (Cook et al., 2023). This is not to say that the groups (cryptophytes, chlorophytes and chrysophytes) associated with softer waters are irrelevant in hard waters, but their relative contributions decline.

The ecological hardness threshold appears to act on a broad spectrum of freshwater algae, with effects on the relative distribution of the main groups and within them, as depicted by diatoms. Different mechanisms can be involved, but carbon uptake for photosynthesis likely plays a central role (Talling, 1976). Cyanobacteria have evolved effective mechanics of CO_2 concentration with efficient bicarbonate transporters that maintain a high CO_2 supply rate to Rubisco in waters where bicarbonate is the dominant form of inorganic carbon (Badger & Price, 2003). Diatoms have also evolved inorganic carbon concentrating mechanisms (Shen et al., 2017), including a biochemical concentration similar to C4 (Hopkinson et al., 2016). At the other extreme of the pH-alkalinity gradient, chrysophytes can only take CO_2 as the inorganic carbon source from the water (Maberly et al., 2009), restricting their dominance to soft waters. Indeed, our study shows that its dominance starts to decline at Ca^{2+} (alkalinity) values ~100 μeq L^{-1}.

Interestingly, a 200 μeq L^{-1} alkalinity ecological threshold was identified by Catalan, Barbieri, et al. (2009), analysing a broad dataset of European mountain lakes in which organisms other than phytoplankton were considered. The benthic diatom communities were the most sensitive to this alkalinity threshold.

4.3 | Trophic state

High mountain lakes show low trophic states due to their headwater character, which limits nutrient loadings. TP is often used to characterize the trophic state with a single variable. In this study, 72% of the lakes were classified as ultraoligotrophic (TP < 4.7 μg L^{-1}), 22% oligotrophic (4.7 < TP < 9.3 μg L^{-1}) and 6% as mesotrophic systems (9.3 < TP < 31 μg L^{-1}) (OECD, 1982). Chrysophytes were the dominant group in ultraoligotrophic lakes and were substituted by cryptophytes as the dominant group at concentrations >5 μg TP L^{-1}. Chrysophytes show a higher affinity for P at very low concentrations (Reynolds, 2006). In a recent field experiment with dissolved P enrichment, chrysophytes showed a response peak at 14 μg P L^{-1}, while cryptophytes, diatoms and chlorophytes increased growth and abundance with greater availability of P (Zufiaurre et al., 2021). In this experiment, the plankton showed stoichiometric homeostasis within the low P-availability range and a high N:P ratio, maintaining cell atomic N:P ratios above 30 and a sudden shift to Redfield proportions (N:P ~ 16) at about 15 μg P L^{-1} when cryptophytes, chlorophytes and diatoms strongly responded (Giménez-Grau et al., 2020). Changes in the phytoplankton group dominance across the P-availability gradient in mountain lakes may respond to the P affinity and differences in stoichiometric homeostasis. N to P imbalances may propagate through the food-web conditioning metazoan life cycles, as shown for zooplankton (Ventura & Catalan, 2008).

Although P limitation is common in many high mountains of the temperate zone, there are areas with N limitation (Williams et al., 2016), particularly when the soil is poorly developed (Saros et al., 2011). These areas could have been more frequent in the past, as many ranges in the Northern Hemisphere have been affected by human-enhanced atmospheric N deposition since the Great Acceleration, including the Pyrenees. In N limitation or N-P co-limitation situations, cyanobacteria could have played a more relevant role. This hypothesis cannot be currently evaluated in the Pyrenees. Therefore, it would be interesting to conduct surveys across other high mountain ranges of the world under different N-P conditions.

Within the trophic gradient considered in this study (ultraoligotrophy-mesotrophy), we found that cyanobacteria peaked at intermediate TP concentrations (~5 μg L^{-1}), reasonably within the oligotrophic range, while the lowest values were found at both extremes of the trophic gradient. This pattern indicates that cyanobacteria are not related to eutrophication processes but constitute an idiosyncratic cyanobacterial assemblage typical of oligotrophic waters (Reinl et al., 2021). GAM did not show TP as a significant factor in cyanobacterial distribution within the high mountain trophic range.

Cryptophytes were the phytoplankton group more closely associated with the TP gradient. Cook et al. (2023) also found alloxanthin, a unique cryptophyte marker pigment, correlated with TP in the Canadian Rockies. Other studies have shown that within oligotrophic water, cryptophytes are the group that exhibits a continued increase in TP (Watson et al., 1997). Cryptophytes are particularly suitable food for zooplankton grazers due to their sterol and ω-3 content (Peltomaa et al., 2017), which could contribute to explaining the close association between TP and MZP density.

4.4 | Food web

The density of MZP increased with the increase of total Chl-*a*, which shows that grazing does not produce a strong depletion effect on phytoplankton levels in these oligotrophic environments, but primary producers and grazers increased proportionally. The relative abundance of chlorophytes was more closely related to MZP than cryptophytes in RDA-6, while GAM showed a more substantial

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independent effect on cryptophytes. This feature can result from selective predation upon cryptophytes, which are known to be preferentially eaten because of their weak cell protection (Reynolds, 2006). On the contrary, gelatinous covers and cellular walls with thick cellulose layers in chlorophytes can make even small forms less edible (Porter, 1973). According to MRT, MZP density (~3000 ind m^{-3}) became a better reference for distinguishing between the ultraoligotrophic domain of chrysophytes and the domain of cryptophytes and chlorophytes. Zooplankton biomass has been related to phytoplankton community composition in the lakes and ponds of the Canadian Rocky Mountains associated with higher alloxanthin (cryptophytes) concentration (Vinebrooke & Leavitt, 1999).

Fish are alien species in high mountain lakes (Ventura et al., 2017). They are the result of human introductions, which, in the Pyrenees, started centuries ago but accelerated during the last decades by stocking for sport fishing (Miró & Ventura, 2013). The selection of fish as a significant explanatory variable in the RDA suggests that fish could drive trophic cascade effects in the Pyrenean lakes. Trout species introduced in high mountain lakes of the Pyrenees are opportunistic species that predate mainly on benthic organisms (Catalan et al., 2004), although they are partially planktivorous and can consume planktonic organisms above a specific size range (Fitzmaurice, 1979). Trout presence in lakes can reduce MZP density (Knapp et al., 2001), which could result in higher algal biomass (Sarnelle & Knapp, 2005). Although Chl-*a* was higher in lakes with trout ($p < 0.05$) in our study, the density of MZP was also higher in lakes with trout ($p < 0.05$). These results suggested no trophic cascade effects from trout to phytoplankton through MZP grazers, although a quantitative estimation of trout density could show more precise results. In some other studies, moderate effects of fish on zooplankton had been found (Loewen et al., 2019) and in other cases climate extremes better explained phytoplankton dynamics than fish-stocking histories (Parker et al., 2008).

Great differences in fish introduction history and stocking intensity in distinct areas of the Pyrenees (Miró & Ventura, 2013) likely resulted in contrasting differences in trout densities within our dataset. On the southern slope of the Pyrenees, trout have been introduced mainly in lower-altitude lakes, those with higher trophic status (Catalan, Barbieri, et al., 2009). Therefore, the higher abundance of MZP in lakes with trout could also result from overlapping factors controlling the abundance of MZP, such as trout being stocked in more productive lakes (lower-altitude lakes). In RDA-6 and GAM, these collinearities were considered. Interestingly, significant independent variance explanation remained associated with fish presence despite including TP and MZP. Some subtle fish effect requires another observational design that better considers the abundance of different fish species and the interaction with lake characteristics. The recent increase in minnows across many lakes could have profoundly changed the situation (Miró & Ventura, 2015).

AUTHOR CONTRIBUTIONS

Jordi Catalan designed the study; Teresa Buchaca and Jordi Catalan collected the data in the field; Teresa Buchaca performed pigment and numerical analyses and wrote the initial draft. Jordi Catalan provided valuable discussion points and revisions to the manuscript.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest to declare.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.xpnvx0kns (Buchaca & Catalan, 2024).

ORCID

Teresa Buchaca https://orcid.org/0000-0001-7933-8992
Jordi Catalan https://orcid.org/0000-0002-2934-4013

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1: Lakes of the Pyrenees considered in the study.

Table S2: Molar ratio between Chl-*a* and marker pigments for the phytoplankton groups considered in the study.

Table S3: Redundancy analysis with forward selection to obtain a minimal explanatory model for each group of environmental variables.

Table S4: Generalized additive models for the relative abundance of the phytoplankton groups.

Figure S1: Chl-*a* estimation of each phytoplankton taxonomic group considered using either a unique sample group of lakes (CHEMTAX 1 group) or two groups of lakes with potentially different pigment ratios (CHEMTAX 2 groups).

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