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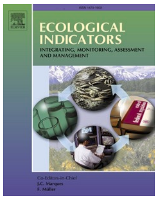

Environmental drivers of genetic variability in common diatom genera: Implications for shallow lake biomonitoring

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ABSTRACT

Diatoms are microalgae, known to present several characteristics that make them excellent bioindicators. In this study, we analyzed the epiphytic diatom communities of ponds, with the aim of exploring the genetic variability explained by physical, chemical or geographical variables. We selected 77 lakes within the Spanish part of the Duero River Basin characterized by wide chemical variability. In each lake, physical parameters were measured *in situ* and water samples were taken to determine nutrient levels in the laboratory. Diatom communities were collected and identified by DNA-metabarcoding, amplifying a genetic barcode of *rbcL gene*. We analyzed the phylogenetic signal for several environmental parameters at the genus level for the three most representative genera of these lakes. Our results indicate significant phylogenetic signals that differ from a genus to another. Processes related to spatial scales appear to be the main predictors controlling the genetic diversity of diatom communities in these ponds, with genetic variants differing among geographical areas for a given species. These molecular studies confirm the enormous diversity present in the diatom communities that inhabit these ecosystems, but further research is needed to apply diatom-based techniques in ponds monitoring or conservation programs. Our results reinforce the view that considering not only morphological but also molecular and ecological characters are necessary to describe new living diatom species, whenever possible, especially given the high number of species described each year.

1. Introduction

Multiple environmental stressors, such as agricultural and industrial runoffs, anthropogenic pollution, and climate change, are threatening riverine habitats worldwide. There is an ongoing international effort to design effective biomonitoring tools for these environments, based on different components of the biocenosis, especially primary producers such as diatoms. In this regard, classical biomonitoring relies on comprehensive metrics, which are in turn based on species abundances and their ecological preferences. However, taxonomists often have problems with defining species boundaries. Since the beginning of diatom taxonomy these boundaries were based only on morphologic characteristics, defined by Cain (1954) as 'morphospecies' and used by Mann (1999) to establish the 'morphological species concept'. However, the development of new molecular techniques such as DNA barcoding combined with high-throughput sequencing (HTS) (Hebert et al., 2003) applied to taxonomy, enable a fine-grain and more objective definition of species in what Dayrat (2005) calls 'integrative taxonomy' to define new species beyond morphological characteristics. For instance, some authors highlighted the need to consider morphological characteristics, molecular data, and ecological characteristics to define species boundaries (Abarca et al., 2020; Carballeira et al., 2017; Kahlert et al., 2019; Trobajo et al., 2013).

Recent phylogenomic studies complemented with morphometry (Çiftçi et al., 2022), attempted to investigate the evolutionary history of the ubiquitous diatom genus *Nitzschia*. Whereas several authors propose new species based on morphological, molecular, and ecological traits (Abarca et al., 2014; Abarca et al., 2020; Carballeira et al., 2017; Jahn et al., 2019; Trobajo et al., 2009), descriptions based only on morphological and ultrastructural characters are still numerous (Trobajo et al., 2013). In recent years, the application of molecular techniques to water quality biomonitoring, has highlighted the problem posed by cryptic diversity (Mann and Evans, 2007), particularly in the definition of ecological preferences among cryptic species (Poulíčková et al., 2017)

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such as taxa within *Navicula* (Poulíčková et al., 2010), *Sellaphora* (Behnke et al., 2004; Evans et al., 2009; Vanormelingen et al., 2013), *Fragilaria*, or *Ulnaria* (Kahlert et al., 2022).

Diatom distribution patterns have been widely analyzed, with studies focusing on local factors in river communities (Rimet, 2012) or dispersal barriers in lakes (Benito et al., 2018; Casteleyn et al., 2010; Verleyen et al., 2009). Several studies discussed the relative contribution of geographical and environmental factors in different ecosystems (Heino et al., 2017) or reservoirs (Marquardt et al., 2018; Zorzal-Almeida et al., 2017) showing that, whereas abiotic constraints seem to be the main drivers at local geographical scales, dispersal barriers were more relevant at larger scales, i.e., across the Andes region (Benito et al., 2018), Europe (Heino et al., 2015; Soininen et al., 2019), or even comparing lake diatom floras between continents (Bennett et al., 2010).

Diatoms show also a noticeable 'niche conservatism' (Keck, 2016; Keck et al., 2016a; Nakov et al., 2015; Romdal et al., 2013; Vanelslander et al., 2009; Verleyen et al., 2009), that is, they 'retain' ancestral ecological preferences (Wiens and Graham, 2005). This was examined through the phylogeny of natural river diatom communities (Keck et al., 2016c) and of diatom cultures during ecotoxicological herbicides tests (Esteves et al., 2017; Larras et al., 2014).

Besides lotic systems, ponds—particularly under a Mediterranean bioclimate—have received relatively little attention, despite their fundamental role in biodiversity conservation (Williams et al., 2004), carbon sinking, or nutrient retention (Hilt et al., 2017; Gilbert et al., 2021) and water supply to populations. Existing diatom indices are not well suited for such environments because of the low number of species considered for calculating the quality metric indices (Borrego-Ramos et al., 2021). In this regard, the question of the taxonomic resolution needed is relevant: while most diatom indices are based on species-level identifications (e.g., Biological Diatom Index - BDI of Coste et al., 2009; Trophic Diatom Index - TDI of Kelly and Whitton, 1995), some studies suggest that genus or even family level can be sufficient (Rimet and Bouchez, 2012; Rumeau and Coste, 1988). Recently, metabarcoding studies used subspecies levels and showed a good accuracy in assessing impairment (Tapolczai et al., 2019a). Hence, there is a balance to be found between bioassessment robustness and the ability to resolve slight differences in pollution levels with diatom biomonitoring tools (Tapolczai et al., 2019b).

In this study, we not only focus specifically on how diatom communities respond to environmental factors, but also to geographical constrains. Previous studies showed that local factors and spatial barriers explain an important part of variability in diatom communities (Bennett et al., 2010), and that variability might also be revealed in genetic diversity. In fact, when the phylogenetic signal has been applied for biomonitoring, successful results have been obtained (Keck et al., 2016a, Keck et al., 2016b). These studies also suggested that it may not provided that phylogenetically-related species have equivalent environmental tolerance thresholds (Carew et al., 2011), and also it may be not necessary to identify DNA sequences up to the species level.

The overall objective of this study was to explore genetic diversities within common freshwater diatom genera and their dependence on local environmental (chemical and physical) factors, geographical barriers, and their phylogenetic niche conservatism.

To do so, we particularly expect that a) intrageneric genetic diversity can be explained by local (chemical and physical) factors and dispersal barriers (geographical distances), b) these factors explain a significant part of genetic variability within common diatom genera, and c) a phylogenetic signal will be detected for such parameters.

We applied this research question to ecosystems delivering important services to local human populations: shallow ponds of the NW region in Spain. To answer these hypotheses, we applied multivariate statistical techniques examining the relationship between diatom communities, physical and chemical parameters, and dispersal barriers among ponds. Redundancy analysis (RDA) with variance partitioning was used to examine environmental filtering and spatial effects on the diatom pond assemblages. Additionally their phylogenetic signal at different taxonomic levels was tested to assess the presence of significant ecological patterns. Phylogenetic signal refers to the tendency of closely related species to have similar ecological characteristics or traits due to shared evolutionary history. Based on the results either meeting or not meeting these assumptions, we would draw a conclusion which could enable an adapted strategy to develop biomonitoring diatom indices for ponds.

2. Materials and methods

2.1. Study site

The study area is within the Duero River basin in the northwest of the Iberian Peninsula, which is in south-western Europe. It has an area of 97,000 km^2, of which 81% is in Spain and 19% is in Portugal. Samples were taken from ponds (Fig. 1; Supplementary Material 1) located between 600 and 1200 m above sea level (m asl) in Spain. Agricultural intensification, rainfed crops which have shifted classification to irrigated crops in recent decades, was suspected to have major impacts on these lakes. The selected lakes cover a wide range of environmental conditions (Table 1).

2.2. Sampling

In total, 77 ponds were sampled during Spring of the period from 2018 to 2021. Diatoms were dislodged from the submerged stems of *Schoenoplectus lacustris* (L.) Palla, or *Typha domingensis* (Pers.) Steud, when *S. lacustris* was absent. In accordance with suggestions from previous studies (Blanco and Bécares, 2006; Blanco et al., 2004), at least 10–12 stems were randomly selected over the pond surface and cut at 10 cm below the water level in each pond. For each pond, the stems were placed together in a 1 L plastic bottle filled to 0.5 L with distilled water. Then, the bottles were shaken for two minutes to detach the diatoms from the stems (Zimba and Hopson, 1997; Riato et al., 2018; Borrego-Ramos et al., 2019). A subsample for microscopy analysis was preserved in 4% v/v formaldehyde, and a subsample for molecular analysis was conserved in 70% v/v ethanol and stored in the dark for further analysis. At the same time, for each pond, a water sample was collected for diatom sampling in order to evaluate the nutrient level. Total nitrogen (mg L^{-1}) and total phosphorus (μg L^{-1}) concentrations were determined following standard procedures (Association, 1989).

2.3. Molecular analysis

Samples collected in 2018 (22 ponds) were treated following the protocol described in the works of Borrego-Ramos et al. (2021) and Nistal-García et al. (2021), summarizing the DNA which was extracted using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). For each DNA sample, six PCR replicates were carried out on 10–20 ng/μL of extracted DNA in a mixture (50 μL final volume) containing 2 U of Platinum™ II Taq Hot-Start DNA Polymerase (Invitrogen, Grand Island, NY, USA), 10 μL of 5 × Platinum™ II PCR Buffer, 0.5 μM of each primer, 5 μL of dNTP mix (2 mM each), 10 μL of Platinum™ GC Enhancer, and 9.6 μL of nuclease-free water. PCR conditions included an initial denaturalization step at 94 °C for 4 min followed by 40 cycles of denaturalization at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 68 °C for 30 s, and a final extension step at 68 °C for 10 min. The libraries preparation and sequencing were carried out by Sistemas Genómicos S.L. (Valencia, Spain).

Samples collected in 2019, 2020, and 2021, were treated according to the following protocol. DNA was isolated from a 2 mL subsample centrifuged at 11,000 G for 30 min. The supernatant was discarded, and the pelleted material was resuspended in nuclease-free water. Extraction was performed using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's

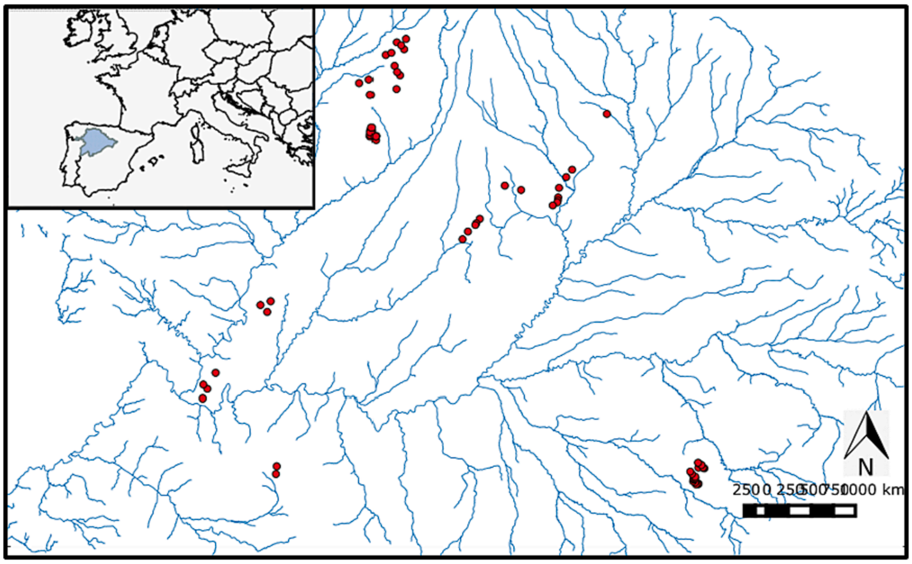

Fig. 1. Location of the 77 studied ponds in the NW of Spain.

Table 1
Environmental variables taken in the studied ponds.

Variable	Mean (range)
Chemical variables	
Total suspended solids (mg L^{-1})	16.05 (1.07–130)
Soluble Reactive Phosphorus (μg/L)	305.47 (1.24–3817)
Chlorides (mg L^{-1})	118.71 (1.38–4045)
Sulfates (mg L^{-1})	17.01 (0.05–137)
Ammonium NH4+ (mg L^{-1})	0.07 (0.008–1.002)
Total nitrogen (mg L^{-1})	1.84 (0.78–4.98)
Total phosphorus (μg/L)	515.97 (9.25–5368)
Chlorophyll (μg/L)	27.71 (1.73–298.10)
Temperature (°C)	20.87(14–27.40)
pH (μg/L)	7.61 (6.21–10.15)
Conductivity (mS cm^{-1})	667.06 (2.88–11460)
Dissolved oxygen (mg L^{-1})	5.69 (0.15–18.68)
Total oxygen (%)	71.67 (1,80–252.1)
Physical variables	
Perimeter (m)	461.64 (58.23–2037)
Surface area (ha)	1.76 (0.02–12.69)
Elevation (m asl)	856.13 (699–1161)

instructions. The gene marker rbcL was amplified by PCR using the primers proposed by Rivera et al. (2018) and Vasselon et al. (2017), an equimolar mix of three PCR primers, 3 forward (Diat_rbcL_708F_1 (AGGTGAAGTAAAAGGTTCWTACTTAAA), Diat_rbcL_708F_2 (AGGTGAAGTTAAAGGTTCWTAYTTAAA) and Diat_rbcL_708F_3 (AGGTGAAACTAAAGGTTCWTACTTAAA)), and two reverse (Diat_rbcL_R3_1 (CCTTCTAATTTACCWACWACTG) and Diat_rbcL_R3_2 (CCTTCTAATTTACCWACAACAG)), including Illumina adapters P5 (CTTTCCCTACACGACGCTCTTCCGATCT) and P7 (GGAGTTCAGACGTGTGCTCTTCCGATC). For each DNA sample, three PCR replicates were performed on 1 μL of extracted DNA in a mixture (25 μL final volume) containing 0.5 μL Phire® Hot Start II DNA Polymerase, 5 μL of Buffer 5X, 2 μL of dNTP mix (2 mM each), 0.25 μM of each primer, and 14.5 μL of nuclease-free water. PCR conditions included an initial denaturalization steps at 94 °C for 4 min followed by 40 cycles of denaturalization at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 68 °C for 30 s, and a final extension step at 68 °C for 10 min. After PCR, the amplification of the rbcL was evaluated using 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized with UV light. DNA metabarcoding libraries and sequencing were carried out by AllGenetics & Biology SL (A Coruña, Spain).

PCRs2 was carried out in a final volume of 25 μL, containing 2.5 μL of DNA from the PCR products received, 1 μM of the dual-indexed primers, 6.5 μL of Supreme NZYTaq 2x Green Master Mix (NZYTech), and ultrapure water up to 25 μL. The reaction mixture was incubated as follows: an initial denaturation at 95 °C for 5 min, followed by 5 cycles of 95 °C for 30 s, 60 °C for 45 s, 72 °C for 45 s, and a final extension step at 72 °C for 7 min. A negative control that contained no DNA (BPCR) was included in every PCR round to check for contamination during library preparation. The libraries were run on a 2 % agarose gel stained with GreenSafe (NZYTech), and imaged under UV light to verify the library size. Libraries were purified using the Mag-Bind® RXNPure Plus magnetic beads (Omega Biotek), following the instructions provided by the manufacturer. Then, libraries were pooled in equimolar amounts according to the quantification data provided by the Qubit™ dsDNA HS Assay (Thermo Fisher Scientific). The pool was sequenced in a fraction of a MiSeq PE300 run (Illumina).

2.4. *Bioinformatic analysis*

Bioinformatic analyses were performed using DADA2 version 1.21 (Callahan et al., 2016, p. 2) following the workflow implemented in diat.barcode package version 0.0.0.9000 (Keck, 2020). Primers were removed with Cutadapt version 3.4 (Martin, 2011). The taxonomic assignment was conducted using the diat.barcode v9 reference database (Rimet et al., 2016; available at https://www6.inrae.fr/carrtel-collection/Barcoding-database/Database-download, accessed in March 2022), and alignment was carried out using Seaview5 (Gouy et al., 2021) and Muscle (Edgar, 2004).

All amplicon sequence variants (ASVs) with more than 10 reads, belonging to Phylum Bacillariophyta, and with a length of 263 bp were kept for the analyses (the others were discarded). In order to avoid the bias introduced by the different sequencing depth among samples, we performed rarefaction using the rrarefy function of the vegan v4.0.4 package (Oksanen et al., 2019) in R. Diatom species codes followed by those given in OMNIDIA software (Lecointe et al., 1993).

2.5. *Statistical analyses*

For the subsequent analysis, three genera with the greatest variability in genetic variants and the greatest number of assigned species of the ASVs were selected: *Gomphomema*, *Nitzschia* and *Achnanthidium*. The spatial variables that made it possible to determine the global spatial structure of the communities were obtained by means of a Principal Coordinates of Neighbor Matrix (PCNM) analysis (Borcard and Legendre, 2002; Borcard et al., 2004). This means that we used this approach to determine whether spatial characteristics had an effect on the heterogeneity of diatom communities in the pond, following the methodology used in previous studies (Rimet et al., 2019). This approach is based on each sampling site's coordinates, determining the Euclidean distance between them. To explore the distribution on variance of the environmental and geographical variables, a variance partitioning was carried out using a forward selection method based on RDA models of each group of variables (chemical, physical, and geographical distances). Redundancy Analysis (RDA) with variance partitioning was used to examine the spatial structure of diatom pond communities. We conducted this analysis using the varpart function in the vegan package in R (Oksanen et al., 2019).

Mantel test and partial Mantel test (Legendre and Legendre, 2013) were carried out to explore the influence of environmental variables or geographical distance on the structure of diatom assemblages, examining the relationship between community dissimilarity matrix and abiotic factors.

Canonical Correspondence Analysis (CCA) was carried out with chemical variables and ASVs to explore the relationship between the environmental variables from the studied ponds and the sequences assigned at species level. All analyses were conducted with R (R Core Team, 2019) using the *vegan* package (Oksanen et al., 2019).

Phylogenetic trees were built based on the substitution model with lowest BIC (Bayesian Information Criterion) using MEGA-X (Tamura et al., 2007). First, we tested the phylogenetic signal at the species level, with the species that had the highest number of sequences. Then, we repeated the analyses with the sequences at the genus level. To do that, the three most abundant genera were selected, so only Amplicons Sequence Variation (ASVs) belonging to *Achnanthidium*, *Nitzschia*, and *Gomphonema* with more than 100 reads were used to calculate the phylogenetic signal. All ASVs present only in one sample were removed. To analyze the response of the sequences variability and the phylogeny of diatoms with respect to abiotic factors, we used Moran's I (Moran, 1948) and Abouheif's Cmean (Abouheif, 1999) indices to test the significance of the phylogenetic signal using the function phyloSignal in the *phylosignal* package in R (Keck et al., 2016b). Moran's I index is the most commonly used autocorrelation measure in spatial statistics. Abouheif's Cmean quantifies the autocorrelation between tips, from a specific matrix of phylogenetic proximities.

3. Results

3.1. Main drivers of diatom communities

MiSeq sequencing generated a total of 2,389,676 raw sequences before any filtering process. After filtering and removing taxa with<10 reads, a total of 2,810 ASVs were taxonomically assigned to 171 diatom taxa belonging to 52 genera from a molecular approach, on which the analysis was performed. The most abundant genera were *Achnanthidium*, *Gomphonema*, and *Nitzschia*, and these were selected subsequently for the phylogenetic analysis. Average read number after bioinformatic analysis per sample was 54,160, with a minimum of 27,425 and a maximum of 141,508 reads. *Achnanthidium minutissimum* (Kützing) Czarnecki was the most abundant species detected after taxonomic assignment, followed by *Nitzschia palea* (Kützing) W.Smith, and *Gomphonema saprophilum* (Lange-Bertalot & E.Reichardt) Abarca et al.

Forward selected variables differed among genera (Supplementary Material 3): For *Gomphonema*, the main parameters selected were

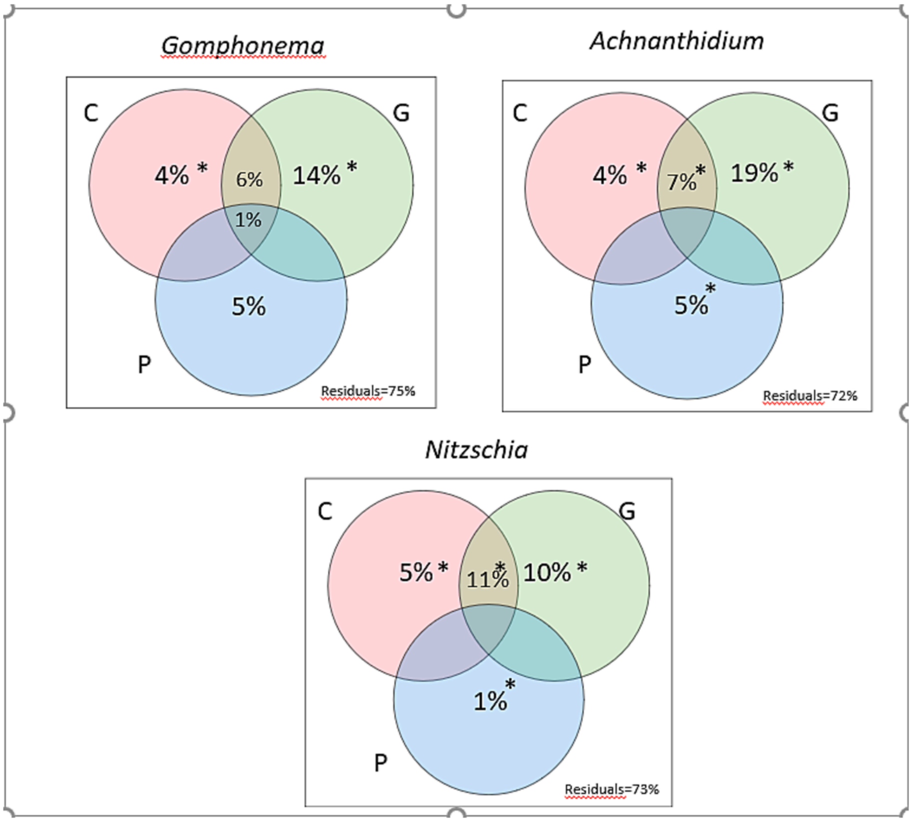

Fig. 2. Results of Variance partitioning into chemical (C), physical (P) and spatial (G) parameters for diatom communities (ASVs) from the studied ponds. Significance level: *: $p < 0.05\%$.

dissolved solids, conductivity, pH, elevation, and geographic distance. In the case of *Achnanthidium*, pH, oxygen dissolved, ammonium concentration, and geographical component were selected, and for *Nitzschia* pH, dissolved oxygen, conductivity, total nitrogen, surface area, and geographic variables were selected.

Fig. 2 shows the results of variance partitioning. Most parts of the variability remained unexplained (ranging from 72 to 75%). However, geographic distance was the main driver of genetic variants within three studied genera: *Achnanthidium*, *Gomphonema*, and *Nitzschia*. Physical variables (which include elevation, perimeter, and pond surface area) explained a lower part of the variance which was, however, significant for *Achnanthidium* and *Nitzschia* genera.

The Mantel test (Table 2) evidences also the role of geographical factors to explain the disparity between communities between ponds. The partial Mantel test shows that the independent effect of spatial distance was also significant, as opposed to the effects of environmental dissimilarities which were negligible.

Canonical correspondence analysis (CCA) performed on environmental data shows that the first two axes explained the 42% and 25% of the total variance, respectively (Fig. 3). The first axis reflected a positive gradient of pH, conductivity and phosphorus concentrations and the second axis was positively correlated with elevation.

3.2. Phylogenetic signal of diatom communities

Moran's I and Abouheif's Cmean indices (Table 3) showed certain parameters having significant phylogenetic signal, again with different responses among the compared diatom genera. Both indices indicate a significant phylogenetic signal in *Gomphonema* for phosphorus concentrations, ammonium, and pH, whereas in *Nitzschia* a significant signal was detected for pH and elevation. Finally, there was a significant signal for suspended solids and pond perimeter for *Achnanthidium*. It is interesting that these genera showed a weaker phylogenetic signal for some factors, as in the case of *Gomphonema* which showed a significant phylogenetic signal related to spatial distribution but only when measured with Abouheif's Cmean.

4. Discussion

4.1. Is there an impact of environmental factors and geographical limitations on diatom communities in ponds?

Our results in the studied pond diatom communities indicate that the intrageneric variability is largely dependent on geographical constraints, which ultimately determine the distribution of genetic variants. Unexpectedly, chemical factors had a negligible effect on diatom communities, in contrast to previous studies at the regional scale based on morphospecies. For instance, pH was found to be the main driver of planktonic diatom assemblages (Bennett et al., 2010) as well as benthic diatom assemblages in high elevation (Feret et al., 2017). Other studies highlighted the role of nutrients in river communities from Sweden (Keck and Kahlert, 2019), in large lakes in Finland (Soininen and Weckström, 2009), or small lakes in Spain (Blanco et al., 2004).

The Mantel test corroborates that geographical variables are the main contributors to inter-lake floristic dissimilitude. Community dissimilarity was typically more strongly correlated with geographic than with physical and chemical factors, in accordance with other studies at large scales (Heino et al., 2017; Virtanen and Soininen, 2012). This evidences that environmental filtering may not account for differences between lake populations (Blanco et al., 2020) because small lake metacommunities are typically organized by species sorting (Szabó et al., 2019) at smaller scales. However, these results were based on diatom communities identified microscopically, whereas our results are derived from molecular identification, with the variability explained by spatial scale factors that were found to be more determinant.

Table 2
Mantel and Partial Mantel tests results comparing the floristic distance matrix with the other distance matrices calculated for Environmental and spatial factors (Geographical distances).

	Matrix	R	p value
Mantel test	Environmental	0.066	0.12
	Geographical	0.346	<0.001
Partial Mantel test	Envir / Geo	0.021	0.62
	Geo / Envir	0.367	<0.001

The structure of large lake diatom communities has been extensively evaluated, seeking key drivers of diatom community composition (Soininen et al., 2019; Soininen and Weckström, 2009). In this study, we address how local and spatial factors modulate pond communities, determining processes related to spatial scales that might be the main predictors of genetic diversity in diatom assemblages. In our case, geographic distance is the main driver of the occurrence of genetic variants within some common diatom genera. However, the large amount of unexplained variance may be related to some unmeasured variables, such as depth (Cantonati et al., 2009; Hudon and Bourget, 1983; Wu et al., 2020), land use (Zorzal-Almeida et al., 2017), or mass effect (Rimet et al., 2022).

4.2. Do the common pond diatom taxa have a phylogenetic signal for some environmental factors?

We did not find a clear phylogenetic signal at species level but rather at genus level, except in the case of *Gomphonema parvulum*. Indeed, our results showed that some environmental variables had a strong phylogenetic signal for certain diatom genera, suggesting that some phylogenetic clades are rather adapted to particular environmental conditions, and that these conditions are probably the results of an ancestral character. The reason that the rest of studied species never exhibited this, could be the short genetic marker used, in this case *rbc*L, even though it is an excellent marker to delimit species (Kermarrec et al., 2013), which might not be adequate to give a clear phylogenetic signal between species of the same genus (Abarca et al., 2020).

For example, only *Gomphonema parvulum* showed a clear phylogenetic signal with respect to TSS. This species shows great genetic and morphological variability, and the significant phylogenetic signal related to geographical distance found in *Gomphonema* (Table 3) evidences the huge diversity of this genus, which is one of the most taxonomically complex genera (Abarca et al., 2020, Abarca et al., 2014), and this is also evidenced by the poor separation between species in the phylogenetic tree (Supplementary Material 4).

The strength of the phylogenetic signal varied depending on the genus. For instance, *Nitzschia* exhibits a strong phylogenetic signal related to pH and elevation. The interpretation of this result is challenging, since most of the *Nitzschia* sequences were assigned to *N. palea*, a species renowned for its great complexity (Trobajo et al., 2009; Mann et al., 2021). In the case of *Achnanthidium*, we observed a strong phylogenetic signal with TSS and pond perimeter. In field experiments dealing with diatom sensitivity to herbicides (Esteves et al., 2017; Larras et al., 2014), *N. palea* showed no intraspecific phylogenetic signal in contrast to *Achnanthidium* species.

Despite the lack of comparable studies with lake datasets, some studies in lotic benthic communities along rivers in France (Keck et al., 2016c), found different signals related to nutrients or pollutants for certain taxa in accordance with their bioindicator value. Similar findings in macroinvertebrates (Buchwalter et al., 2008) or fishes (Hylton et al., 2018) demonstrate that phylogenetic signal explains, to a large degree, the different species sensitivity to environmental stressors. However, ecological and phylogenetic similarities cannot always be related (Losos, 2008). In fact, the idea of phylogenetic niche conservatism would suggest the use of higher taxonomic ranks as good surrogates to species

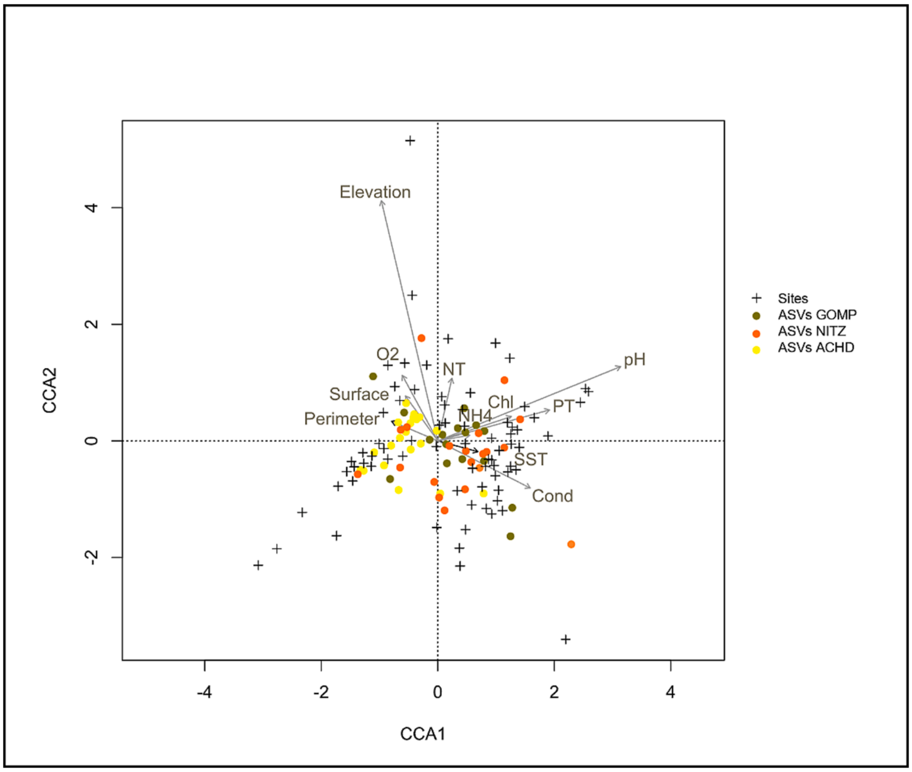

Fig. 3. Canonical Correspondance Analysis of environmental factors and the Amplicon Sequence Variants (ASVs) assigned to *Gomphonema* (GOMP), *Achnanthidium* (ACHD) and *Nitzschia* (NITZ).

level in order to assess anthropogenic impacts on aquatic ecosystems (Keck et al., 2016a; Webb et al., 2002).

4.3. Application of molecular methods for biomonitoring?

Limitations of the application of molecular methods for biomonitoring of aquatic environments have been widely studied (Borrego-Ramos et al., 2021; Kelly et al., 2020; Mortágua et al., 2019; Pérez-Burillo et al., 2020; Rivera et al., 2020). Those studies, which included rivers, lakes, and ponds, revealed a large concordance in results when applied to water quality biomonitoring. Although the results are quite reliable, they complement the information provided by traditional microscopy-based protocols. For instance, some species cannot be distinguished under a light microscope, due to the limitations of this method. Certain taxa may be overlooked by microscopic analysis, such as *Fragilaria flavovirens* Takano, probably due to their weakly silicified valves. In the case of *Mayamaea*, their poorly silicified valves explain their absence after the digestion process with hydrogen peroxide and hydrochloric acid (Kelly et al., 2020; Zgrundo et al., 2013). Thus, despite the fact that the methodology is already well advanced, there is still room for further improvements before their implementation as monitoring standards (Blancher et al., 2022; Leese et al., 2018), some of these problems being related to cryptic species taxa or species complexes taxa. In this sense, the generic concept in diatoms is currently much constrict. The number of genera has doubled in recent years, and the ecological preferences of each are better defined. Today, diatom indices constructed from a genus-level taxonomy can be effective for large-scale assessments (Riato et al., 2022). Many studies are finding that the use of genus-level diatoms produce a similar or greater diatom-environment relationship than at the species level (Kilroy et al., 2017). Genus-level communities tend to be more homogeneous among regions than at the species level, and this homogeneity increases their usefulness as ecological indicators (Rimet and Bouchez, 2012). Many other studies reach similar conclusions, both in rivers and lakes, regardless of the environmental gradient reflected by the index (Hill et al., 2001; Lane, 2007).

5. Conclusions

Metabarcoding techniques aid in dealing with well-known issues affecting biomonitoring studies, such as cryptic diversity. Although the idea of cryptic species originated years ago, it has become evident with current genetic studies, and many inconsistencies have arisen with respect to ecological preferences, which are the basis for the estimation of water quality. Therefore, when taxa used in biomonitoring studies are actually species complexes (Abarca et al., 2020; Pinseel et al., 2017a, Pinseel et al., 2017b), it becomes necessary to acknowledge this complexity when defining new metrics based entirely on molecular data.

This study contributes to the understanding of the processes occurring in small Mediterranean lakes, which largely differ from large European temperate lakes in many features. Molecular studies confirm the huge diversity present in diatom communities inhabiting these ecosystems, but further studies are needed to meet the challenge of implementing diatom-based techniques in monitoring or conservation programs. Since global aquatic biodiversity is declining rapidly, this type of study describing the structure of these important communities can contribute to our knowledge and promote future studies.

The contrasting sensitivity shown by different diatom genera present opportunities for developing trustworthy biomonitoring technologies based on predictive sensitivity. It leads to the question whether taxa boundaries are properly defined. Our results reinforce the view that to both describe new species and solve taxonomic complexes it is necessary to consider not only morphological but also molecular and ecological characters.

Table 3

Test of phylogenetic signal in environmental variables for the genera *Achnanthidium*; *Gomphonema* and *Nitzschia*, and the species *Gomphonema parvulum*. Moran's I and Abouheif's Cmean are reported for each environmental parameter. p values are shown between brackets. Significance level: +: $p < 0.1$; *: 0.05; **: $p < 0.01$; ***: $p < 0.001$; ns: non significant.

Gomphonema	Abouheif's Cmean	Moran's I
TSS	0.001 (ns)	0.005
SRP	0.340 (***)	0.030 (**)
NH4	0.338 (**)	0.027 (**)
Total nitrogen	0.147 (**)	0.004 (ns)
Total phosphorus	0.321 (***)	0.025 (**)
Chlorophyll	0.136 (**)	0.006 (ns)
pH	0.124 (*)	0.011 (**)
Conductivity	−0.014 (ns)	0.007 (*)
Total oxygen	−0.093 (ns)	−0.020 (ns)
Elevation	0.111 (**)	0.001 (ns)
Perimeter	0.064 (ns)	−0.012 (ns)
Geographical	0.141 (*)	0.008 (ns)
Nitzschia		
TSS	−0.001 (ns)	−0.007 (ns)
SRP	0.157 (**)	0.010 (ns)
NH4	0.021 (ns)	−0.002 (ns)
Total nitrogen	0.074 (ns)	−0.004 (ns)
Total phosphorus	0.162 (**)	0.012 (ns)
Chlorophyll	0.077 (ns)	0.002 (ns)
pH	0.219 (***)	0.032 (**)
Conductivity	0.010 (ns)	−0,001 (ns)
Total oxygen	−0.092 (ns)	−0,022 (ns)
Altitude	0.165 (**)	0,018 (*)
Perimeter	0.054 (ns)	−0,006 (ns)
Geographical	0.04 (ns)	0,009 (ns)
Achnanthidium		
TSS	0.109 (*)	0.005 (*)
SRP	0.003 (ns)	−0.009 (ns)
NH4	0.118 (*)	−0.002 (ns)
Total nitrogen	0.069 (ns)	−0.007 (ns)
Total phosphorus	0.028 (ns)	−0.007 (ns)
Chlorophyll	0.089 (ns)	0.003 (*)
pH	0.046 (ns)	−0.002 (ns)
Conductivity	0.068 (ns)	−0.001 (ns)
Total oxygen	0.071 (ns)	−0.001 (ns)
Altitude	0.039 (ns)	−0.005 (ns)
Perimeter	0.138 (**)	0.009 (**)
Geographical	−0.064 (ns)	−0.012 (ns)
Gomphonema parvulum	**Abouheif's Cmean**	**Moran's I**
TSS	0.379 (***)	0.09 (*)
SRP	−0.02 (ns)	−0.05 (ns)
NH4	0.004 (ns)	−0.079 (ns)
Total nitrogen	−0.296 (ns)	−0.143 (ns)
Total phosphorus	0.049 (ns)	−0.026 (ns)
Chlorophyll	−0.095 (ns)	−0.065 (ns)
pH	0.197 (ns)	0.022 (ns)
Conductivity	0.002 (ns)	0.046 (ns)
Total oxygen	0.066 (ns)	−0.058 (ns)
Altitude	−0.156 (ns)	−0.09 (ns)
Perimeter	−0.1 (ns)	−0.166 (ns)
Geographical	0.379 (***)	0.09 (**)

CRediT authorship contribution statement

María Borrego-Ramos: Data curation, Formal analysis, Writing – original draft. **Frédéric Rimet:** Conceptualization, Formal analysis, Writing – review & editing. **Eloy Bécares:** Funding acquisition, Conceptualization, Writing - review & editing. **Saúl Blanco:** Conceptualization, Methodology, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2023.110898.

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