CHARLES UNIVERSITY
Faculty of Science

What can morphology tell us
about the evolution and ecology of diatoms?

Habilitation thesis

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1. BACKGROUND

1.1. General information about diatoms

Diatoms (Bacillariophyta) are unicellular algae (solitary or colonial) that inhabit different types of aquatic habitats [1, 2]. They occur in almost every drop of natural water where abiotic conditions do not prevent the life of eukaryotic oxygenic organisms[1], and where photosynthesis is not hampered by a lack of light for extended periods of time. Diatoms are an important component of pelagic communities (plankton) and of substrate-associated biota (benthos), the latter of which can be further divided into the epilithon (biofilms covering bedrock, rocky littoral zone, wet walls), the periphyton (loosely attached among filamentous algae or macrophytes), and the epipelon (soft sediments). In these attached communities, raphid pennate diatoms can move using mucilage[2] secreted through a slit in their cell wall called the raphe [1-3]. The importance of diatoms lies not only in their high primary production in marine, brackish, and freshwater ecosystems supporting variable food webs, but also in their significant contribution to the global biogeochemical cycles [4, 5].

Diatoms are easily recognized from other stramenopile algae by their **perforated siliceous cell walls** (frustules) composed of two parts[3] that fit together like a Petri dish [1]. The origin and evolutionary diversification of diatoms can be dated relatively easy because pieces of their cell walls found in paleosediments can be used to calibrate the geological time scale. Diatoms most likely evolved in shallow shelf seas in the Mesozoic or late Permian [2, 6], from a common ancestor they share with the Bolidophyceae [7]. The close relationship of diatoms to these picoplanktonic marine flagellates, which have no siliceous structures, has been difficult to explain because it has been assumed that the common ancestor had siliceous structures at least for some part of its life cycle [8]. This assumption regarding siliceous structures was fulfilled by the discovery that a non-motile coccoid nanoplankton with siliceous plates belonging to the genus *Triparma* (formerly part of the Chrysophyta, [9]) is a life stage of the genus *Bolidomonas* Guillou & Chrétiennot-Dinet [10].

Diatoms have diversified in many ways during their evolution: differing in morphology, life history, and ecology [1, 8]. They initiated their evolution as oogamous **centric diatoms** (radial pore arrangement) living in planktonic and benthic habitats of the seas, from which several lineages later invaded into freshwater habitats [11]. Different cell shapes, ranging from radial and multipolar (Figure 1A) to elongated bipolar, have been used to classify centric diatoms, but molecular methods revealed that similar shapes have evolved multiple times during evolution [12-16]. An evolutionary novelty was the emergence of non-motile (araphid) pennate diatoms (Figure 1B) and motile (raphid) pennate diatoms (Figure 1C-I)

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[1] e.g., extremely high temperatures, extremely low pH, anoxic conditions
[2] strands composed of extracellular polymeric substances associated with the cytoskeleton
[3] the name 'diatom' comes from the Greek word 'diatomos', and means 'cut in half'

during the Cretaceous [17]. Both groups of **pennate diatoms** have pores arranged in rows, reproduce by means of gametangia generated by the transformation of whole protoplasts (no flagellates are produced), and have diversified primarily in marine and freshwater benthic environments [11, 12]. Similarly to centrics, common morphological characters in
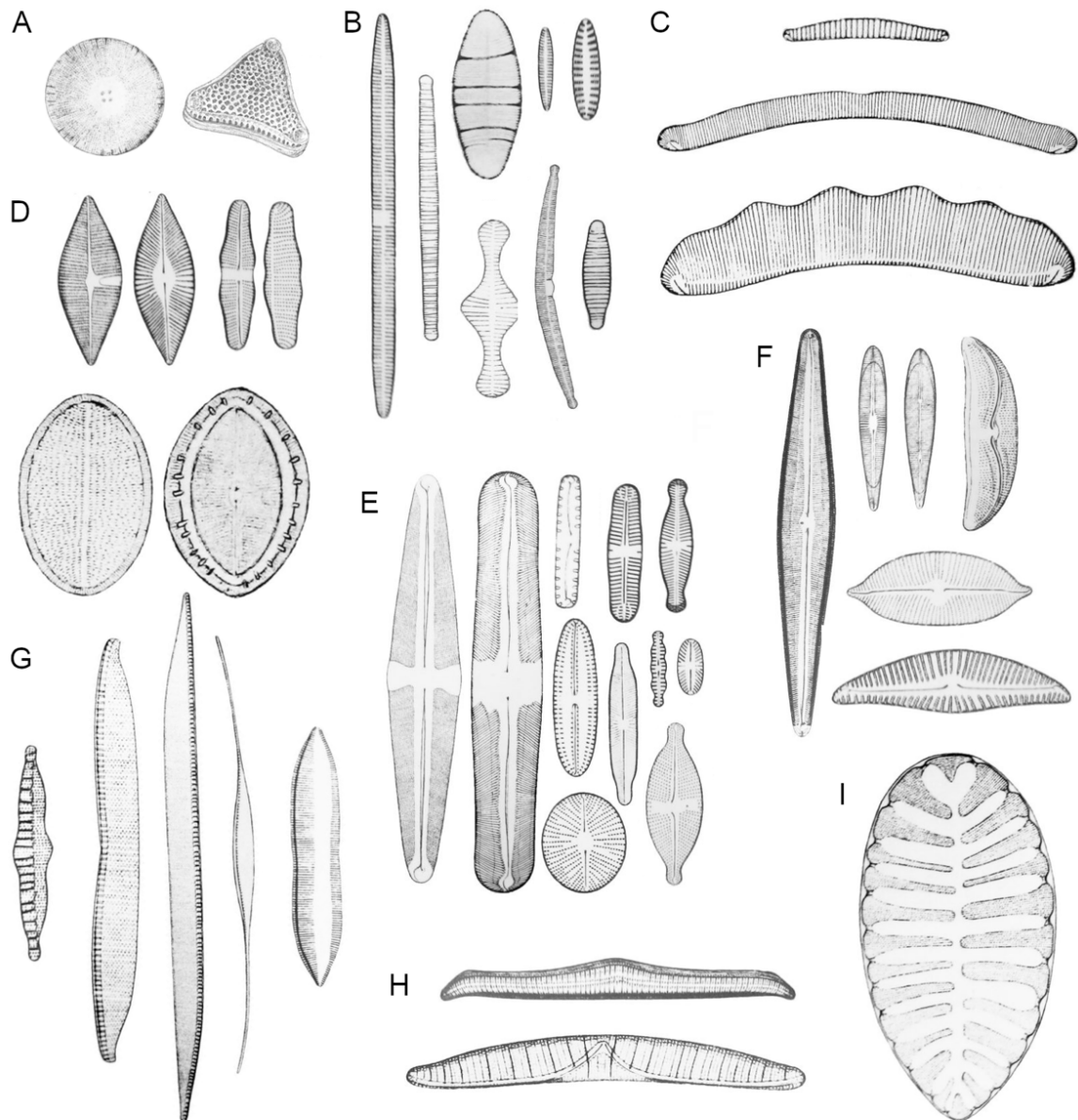
Figure 1. Variability of diatom shapes and forms. **A**: centric diatoms with a radial and tripolar shape; **B**: araphid pennate diatoms of various shapes; **C-I**: raphid pennate diatoms with different type of raphe and symmetry (adapted from https://diatoms.org and https://micro-biologysociety.org).

pennate diatoms (e.g., cell shapes) do not always imply a common ancestry; **convergent and parallel** evolution are detectable at different levels of taxonomic resolution: species [18-21], genera [22, 23], orders [24] and families [25]. There are several - not necessarily mutually exclusive - explanations for morphological convergence in phylogenetically distant taxa: i) the divergence may be limited by intrinsic factors related to their siliceous

cell walls[4] [1, 2]; ii) similar characters may differ in their morphogenesis [22, 26, 27]; and iii) ecologically advantageous morphologies may evolve repeatedly, such as the radial shapes of diatoms living in planktonic habitats [28] and asymmetric shapes of cells attached to substrata [2].

The life cycle of diatoms is unique in several ways because of their bipartite silica cell walls, which are reused during the vegetative phase. The upper part (epitheca) is slightly larger than the lower part (hypotheca; Figure 2A) and both parts are connected by siliceous 'girdle bands' [1]. With each cell division, the two parts move apart to form a new hypotheca, so that both parts of the parental cell walls become the epitheca of the daughter cells [1, 29]. This mode of vegetative reproduction leads to a gradual **diminution of cell sizes in the dividing population** (Figure 2B, C), as the number of smaller cells increase more rapidly than larger cells [30] and the parental parts of the cell wall can be reused for a limited number of cell divisions [31, 32]. Cell size restoration in natural populations is
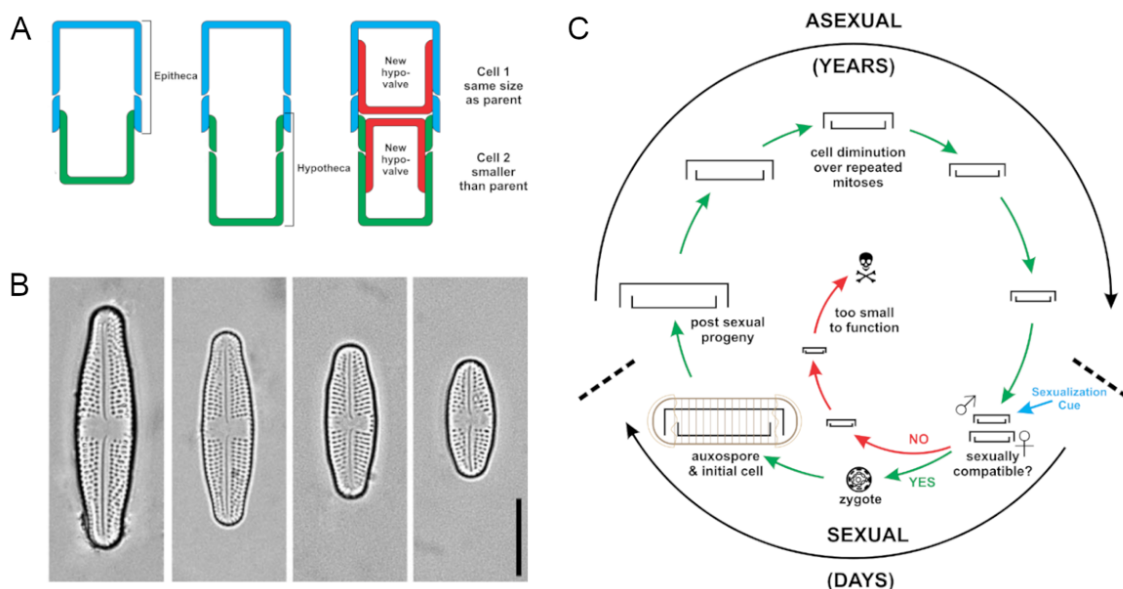
Figure 2. Simplified life cycle of pennate diatoms. **A**: after mitotic cell division, bipartite cell walls consist of a parent and a daughter part (adapted from [29]); **B**: size reduction in *Luticola* clonal culture during the asexual phase of the cycle (adapted from Article 9); **C**: alternating asexual and sexual phases; the sexual phase occurs at a specific cell size and leads to restoration of the maximum size (adapted from [29]). Scale bar = 10 μm.

usually accomplished through sexual reproduction (Figure 2C), which occurs once every two to forty years [8]. The period between two sexual processes can be extended by increasing the size vegetatively (see [8] for details) and manipulating cell size through division rate [33].

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[4] e.g., evolutionary stable morphogenesis, rigid glass-like cell wall

The morphological variability of diatom cell walls has attracted human attention since the 19th century, when the principles of diatom systematics and taxonomy were established [34]. The intensive interest in reporting the occurrence of diatoms at various sites during the 19th and 20th centuries has made it possible to assign autecological characteristics (optima and tolerances) to diatom species that have been used to track recent or past changes in salinity, temperature, trophic level, acidity, and water column depth. Diatom frustules are also important in forensic science [35], biotechnology [36], and industry [37]. Negative societal impacts of diatoms may be caused by their blooms: they can produce toxins affecting vertebrates in coastal areas and cover freshwater stream beds with nuisance mucilaginous mats [2].

1.2. Identification of diatom species - a unified species concept

For more than a century, the identification of diatom species has been based solely on the morphological features of their siliceous cell walls, which can be distinguished using light or electron microscopy. Over the course of history there have been changes to diatom species concepts [34, 38]. 'Lumpers' consider variation in morphologically similar taxa as phenotypic plasticity, while 'splitters' consider it to be interspecific variation. **Species boundaries**, which rely on size, shape, and patterns on empty cell walls, were therefore mostly subjective and dependent on authorities that broadened or narrowed species boundaries.

A less subjective view of diatom species boundaries (before the advent of DNA sequencing) was provided by studies statistically and experimentally analyzing diatom populations. These studies show that many morphologically defined species (morphospecies) have overlapping species boundaries and cannot be unambiguously identified [39]. Some morphospecies may be composed of populations differing in fine morphology, sexual compatibility, and/or ecophysiology [38] while characters of other morphospecies have been erroneously considered interspecific due to their high morphological variability caused by life cycle changes and/or environmental conditions [38, 40, 41].

The diatom species concept underwent a significant rethinking with the molecular revolution in the late 20th century. It has become apparent that some morphospecies are not genetically uniform and that this genetic variation is not always accompanied by morphological differentiation, even after examination of cell wall ultrastructure using electron microscopy [42]. The application of morphometric methods to diatoms has sometimes led to the discovery of morphological differentiation between genotypes [42, 43], so-called pseudocryptic species *sensu* Mann and Evans [44]. More often the extent of morphological variation partially overlapped, which did not allow separation of all members having the same genotype (i.e. 'semicryptic species'; [45, 46]), or overlapped

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entirely and the genotypes remained cryptic [23]. Another problem lies in the choice of molecular marker, because the same molecular marker used in different evolutionary groups of diatoms may tell different evolutionary stories. For this reason, it is not possible to use a single region to identify diatoms by metabarcoding [47-49].

Some diatomists have adopted the so-called **unified species concept** of de Queiroz [50] to acknowledge genetic variation in the absence of unique morphological characters for species identification (e.g., [34, 45, 51, 52]). The unified species concept does not regard one property as superior to the others but considers all evidence of evolutionary divergence of metapopulations[5] as equally important, because trait evolution does not proceed equally across species. For example, two metapopulations cannot interbreed (biological species concept) and form two monophyletic lineages (phylogenetic species concept) but are morphologically indistinguishable (morphological species concept), and two other metapopulations are morphologically distinct and live in a different habitat type (ecological species concept) but can interbreed. All of these populations can be viewed as separate species if the unified species concept is accepted, though speciation is supported more strongly the more evidence there is for divergence.

Given the estimate that there are 100,000 - 200,000 diatom species with approximately 12,000 already described morphologically [53, 54], taxonomic revisions, and cataloging of new species diversity, might take centuries at current rates [55]. This difficulty is further exacerbated by the nature of the polyphasic approach[6], as re-evaluation of the properties of morphospecies by examining strains from different populations takes a long time and may produce inconsistent results [34, 56]. The situation in assigning traditional diatom names to phylogenetic lineages is further complicated by the lack of DNA from type specimens [55].

1.3.   Ecological requirements and distribution of diatoms - molecular view

The use of diatoms as bioindicators has been based on autecological information that has been obtained empirically through the identification of morphospecies in various field samples. With increasing discoveries of hidden genetic diversity within widely distributed diatoms [54], the need to re-examine the geographical ranges, and ecological niches, of traditional species has grown (e.g., [19, 57-59]).

The most common methods for re-examining the distribution and ecology of traditional species utilize Sanger sequencing of several molecular markers from isolates [19, 57] or environmental DNA (eDNA) sequencing using high-throughput sequencing

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[5] a population composed of local populations with limited dispersal/migration between them
[6] the use of multiple approaches to study populations, i.e., molecular methods, morphometric techniques, breeding experiments, and/or cultivation under controlled conditions

methods [59, 60]. Both molecular methods have its limitations. Evidence based on Sanger sequencing is limited by the small number of sequenced strains, which represent only a small fraction of the diversity of natural populations [61]. In contrast, eDNA sequencing generates thousands of sequences, but does not provide direct information on lineage morphology. Traditional taxa are assigned to eDNA sequences using reference databases (e.g., [62, 63]), whose contents are far from complete.

Across marine, freshwater, and terrestrial habitats, both **DNA-based approaches have confirmed patterns** that have been derived from observations of traditional morphospecies. Some genotypes are widely or globally distributed, while others have a scattered or restricted distribution [57, 64]. Some genotypes are confined to relatively stable conditions with respect to temperature (occurrence in a particular season and/or climatic zone, [58, 60, 65]), salinity (intolerance of freshwater conditions, [19, 42]), and trophic status of the waterbody [58, 59, 66]; other genotypes appear to be ecological generalists. At first glance, genotype identification appears to be nothing more than the use of more variable input data that eventually reveals similar ecological patterns. Yet, the ability to evaluate the evolution of traits using phylogenetic reconstructions is an undeniable advantage of DNA sequences. With an understanding of evolution's convergence and parallelism, it is possible to look for mechanisms that lead to speciation and community assembly without becoming confused by dealing with non-monophyletic taxa.

1.4. Subtle shape variations in diatoms - ontogeny and ecology

The recognition of morphological species boundaries in diatoms is complicated by phenotypic plasticity and allometric life history changes [38]. While morphological variability induced by variation in the environmental conditions is usually ignored in biodiversity studies, allometric shape changes are depicted as a series of progressively shrinking cells and verbally described using qualitative descriptors and size measurements. The qualitative description of shape was the only approach until the development of so-called **geometric morphometrics**, which describes shape variation quantitatively [67, 68]. Although geometric morphometrics is roughly the same age as molecular methods, it is not widely used in analysis of diatom cell walls (reviewed in [69]).

Geometric morphometrics is a powerful statistical tool that allows: i) visualizing and testing similarities between groups of objects; ii) reconstructing group-specific shapes; iii) obtaining descriptors of shape and size (e.g., circularity[7], surface area); and iv) testing the success of automatic identification tools [69]. In particular, the latter application has received much attention in diatomology due to its potential use in routine biomonitoring, leading to the development of specific softwares such as ADIAC (Automatic Diatom

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[7] to what extent the shape of the object corresponds to a circle

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Identification And Classification, [70]), SHERPA (SHapE Recognition, Processing and Analysis [71]), and DiaCurv (Diatom Curvature, [72]).

There are basically two morphometric techniques used to describe diatom shapes geometrically: extraction of cell outlines (used in ADIAC and SHERPA), which looks for curves best matching the shape of objects; and **landmark-based morphometrics**, where data are obtained from the positions of points non-randomly placed along the outline of objects [69]. Landmark-based morphometrics, unlike outline-based approaches, can decompose shape variability into different components - allometry, disparity, and asymmetry of symmetric structures - through homology (landmarks) or partial homology (semilandmarks) of points [73]. The basic procedure for landmark alignment, called generalized Procrustes superimposition, consists of three steps: the shapes are rescaled to unit size, translated to the center, and rotated to best fit (Figure 3A; [74, 75]). These transformations allow for shape analysis while ignoring differences in object size, position, and orientation.
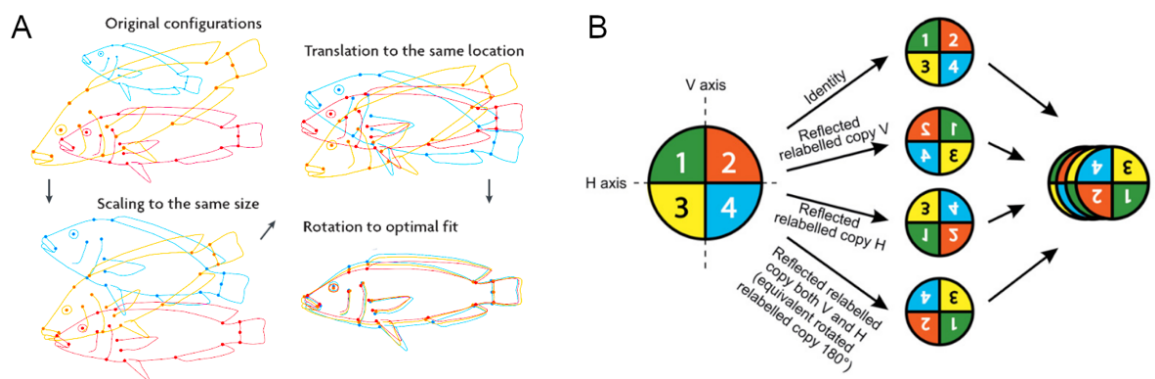

Figure 3. Steps of landmark-based geometric morphometrics. **A**: Procrustes superimposition of landmark coordinates (adapted from [75]); **B**: comparison of symmetric parts in an analysis of asymmetric and symmetric variability (adapted from [80]).

The allometric component of shape variability is calculated by multivariate regression of Procrustes-aligned data on centroid size[8] and can be useful in finding species-specific features that may be masked by the cell size diminution of diatom populations during the vegetative phase of their life cycle [76]. **Disparity** is the quantification of shape diversity based on the relative distances between the coordinates of an object and the coordinates of a reference. It can be used as a measure of biodiversity that is not captured by species identification [77-79]. Mirror reflection of points along one or more axes of symmetry can be used to obtain **asymmetric components** of variability by a quantitative comparison of the original and mirror-reflected coordinates (Figure 3B; [73, 80]). Comparisons of whole-object asymmetry with the data set's average configuration are used to calculate

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[8] Euclidean distances of points from the central part of the object calculated before superimposition

directional asymmetry[9], which is related to evolutionary stable asymmetry [81]. This type of asymmetry is present in pennate diatoms; their symmetrical cell shapes are formed asymmetrically[10] and the internal components may show asymmetry in organelle position, raphe curvature and/or cell wall perforations [27, 82]. Analysis of within-object asymmetry can be used as a measure of fluctuating asymmetry that may reflect developmental instability during cell morphogenesis due to stress, interbreeding, and/or hybridization [83]. The strong influence of stress on morphogenesis is especially visible in diatoms living in toxicogenic conditions that result in the severely malformed cell walls (reviewed in [84]).

2. OBJECTIVES OF OUR RESEARCH

Diatom communities are important components of many aquatic ecosystems, and their composition varies depending on environmental conditions. The previous chapters demonstrate that the degree of morphological diversification in diatoms cannot be used universally because morphological convergence and phenotypic plasticity can obscure the evolutionary signal in different ways. Compared to centric diatoms living in marine plankton, our studied taxa - freshwater benthic pennate raphid diatoms - are the youngest [12] and have high diversification rates due to their ability to actively move (sliding across the substrate), mode of sexual reproduction (prevailing anisogamy), and life in unstable habitats (patchiness in the landscape, local heterogeneity, temporal instability; [11, 85]). They are therefore an appropriate model for tracking microevolutionary changes associated with the current distribution of diatoms.

Using a polyphasic approach, we investigated genetic diversity (sequencing of DNA regions), morphological differentiation (morphometrics of strains and field populations), ecological preferences (abiotic conditions at sampling sites), and geographical range (sampling in various countries) within morphologically similar diatom groups. We used **three widely distributed species complexes** - *Navicula cryptocephala* Kützing (**Article 1**; Figure 4A), *Frustulia rhomboides* (Ehrenberg) De Toni (**Articles 2-5**; Figure 4B), and *Pinnularia borealis* Ehrenberg (**Article 6**; Figure 4C) - that live in different types of habitats: epipelon in alkaline standing waters, periphyton in acidic wetlands, and semi-terrestrial[11] habitats, respectively. These habitats differ not only in their physicochemical properties, but also in their landscape connectivity (semi-terrestrial habitats > alkaline epipelon > acidic wetlands). Our aim was to determine whether morphospecies form monophyletic units and whether genetic diversification is supported by other lines of evidence. We hypothesized that evolutionary forces would affect lineages of our species complexes

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[9] a consistent left/right asymmetry (typically inherited) shared by the majority or all members of a
  population, e.g., the left side of mammalian heart is normally larger than the right side
[10] along the apical axis, the primary side starts to form earlier than the secondary side
[11] top layer of soils, wet or submerged mosses, littoral of ephemeral pools

differently due to differences in their dispersal abilities, breadth of ecological niches, and/or degree of morphological differentiation. The similarity in findings across our species complexes could indicate more general microevolutionary patterns that apply across freshwater environments.
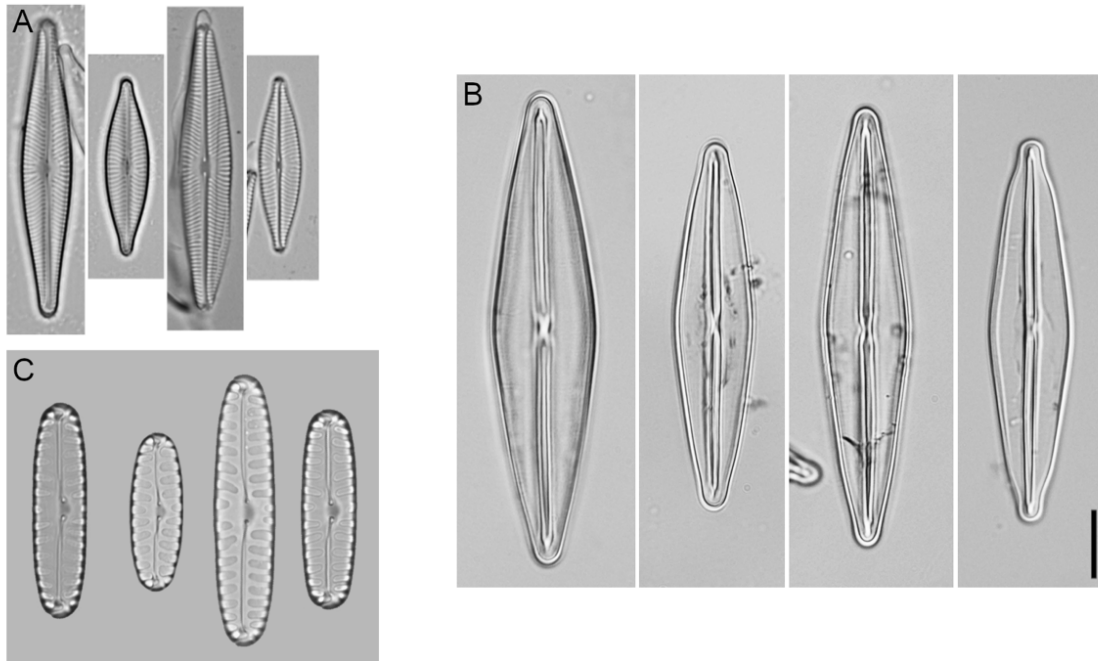

Figure 4. Light microscopic images of the lineages of our species complexes (two cells of different size from each lineage). **A**: *Navicula cryptocephala* (lineages I and II *sensu* Article 1, images from [86]); **B**: *Frustulia rhomboides* (*F. crassinervia-saxonica* lineages VI and V *sensu* Article 2, images from the same source), **C**: *Pinnularia borealis* (lineages D and C *sensu* Article 6, images from the same source). Scale bar = 10 μm.

Traditional species included in our complexes were chosen not only on the basis of morphological similarity, but also on intraspecific names used during the era of coarse morphological species boundaries in taxonomy. In the 1950's, Lothar Geitler published studies on the life cycle of *Navicula* populations (for references see [86]), which we could only partially assign to contemporary species (i.e., *N. cryptocephala*, *N. trivialis* Lange-Bertalot, *N. veneta* Kützing). The remaining species of the complex (N*. cryptotenella* Lange-Bertalot, *N. gregaria* Donkin) were chosen based on morphological similarity. In the case of the *Frustulia rhomboides* species complex, we adopted its broad concept *sensu* Krammer and Lange-Bertalot [87]. The currently used fine-grained taxonomy no longer identifies any populations as *F. rhomboides*, because the varieties have been elevated to species [88] and the type material of *F. rhomboides* does not contain any cell of this genus [89]. Our reason for the original conception was that the name *F. rhomboides* had been used since 1853 to name populations with a lanceolate shape living in oligotrophic acidic habitats (see [90] for details) and that there are unclear morphological species boundaries between the varieties elevated to species and the newly described species [91, 92]. The historical legacy for the

broad sense of *Pinnularia borealis* is the description of more than sixty intraspecific taxa, some of which have overlapping morphological boundaries (**Article 6**).

The second topic of our research was focused on **ontogenetic and ecological signals in morphology of pennate diatoms above the species level**. We examined morphometrically clonal strains of four genera (*Frustulia* Rabenhorst, *Luticola* D.G. Mann, *Navicula* Bory, *Sellaphora* Mereschowski; **Articles 8-10**) and natural populations (**Articles 11-13**) to quantify ontogenetic variation (allometric shape changes) and the effect of environmental conditions (cell shapes and sizes of natural populations). Our aim was to find general trends in diatom morphology that would reflect environmental heterogeneity without neglecting life history changes.

3. RESULTS AND DISCUSSION

3.1. Microevolution of our species complexes

Genetic diversity of morphologically similar taxa

Our first step in the polyphasic investigation of diatom species complexes was the isolation of monoclonal cultures from field samples and sequencing of different parts of their DNA (for our methodology see **Articles 2, 3, 6**). Using universal or own newly designed primers, we obtained partial sequences of nuclear (rRNA small subunit gene - SSU, ribosomal internal transcribed spacer region - ITS, rRNA large subunit gene - LSU), plastidial (RuBisCo large subunit gene - rbcL) and/or mitochondrial DNA (cytochrome oxidase subunit 1 gene - COI). Consistent with barcoding studies [47-49], our **molecular markers had different resolution power**. The most conservative region was the SSU, which did not resolve some morphospecies (**Article 3**). Higher variability was exhibited by the rbcL and LSU regions, which were either comparable in resolution (**Article 3**; [49]) or one showed much higher divergence than the other (rbcL > LSU in the genus *Achnanthidium* [93], rbcL < LSU in *Pinnularia* [94]). The most variable markers were ITS and COI, but both were problematic; ITS showed relatively high intraclonal variation (**Articles 1, 3**; [56]) and COI lacked conserved regions that are necessary for the development of primers applicable across lineages (**Article 3**; [47]).

To delineate lineages (typically composed of several slightly different genotypes), we used the markers rbcL and LSU, which are, however, less prone to microspeciation events than COI [95] and microsatellites [96, 97]. Once we had lineages, we examined the morphology of several strains to give the lineages the names of the traditional taxa or describe species new to science. Some lineages showed a unique morphology, while others could not be unambiguously identified (**Articles 1-6**; [98]).

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Ecology and distribution of recovered lineages

It was important for us to isolate multiple strains from the same sampling site in order to infer the distribution and ecology of lineages because sympatric lineages can vary greatly in abundance [99] and rarer lineages may go undetected. Without collecting samples outside of continental Europe, research on the *Navicula cryptocephala* complex could lead to the conclusion that morphologically similar traditional species (*N. cryptocephala*, *N. cryptotenella*, *N. gregaria*, *N. trivialis*, *N. veneta*) lack hidden genetic diversity and form separate but not phylogenetically most closely related lineages. We would most likely find only one *N. cryptocephala*-like lineage (lineage I *sensu* **Article 1**), while the second lineage (lineage II) would have remained undetected due to its rarer occurrence (one isolate from the Czech Republic versus twelve isolates from Germany, Czech Republic, and Austria). After sampling from more distant areas, both *N. cryptocephala*-like lineages were subsequently found in two of the three locations (once in sympatry - Australia and once alone - Scotland). The ecological requirements of the linages could not be estimated based on our sampling.

Sequencing of *Frustulia* isolates confirmed the monophyly of many nominal species (Figure 5; **Articles 3, 5**) described and reported from either **Holarctic** (*F. erifuga* Lange-Bertalot & Krammer, *F. gaertnerae* Lange-Bertalot, *F. krammeri* Lange-Bertalot & Metzeltin, *F. septentrionalis* Lange-Bertalot) or **Australasia** (*F. aotearoa* T.Beier & Lange-Bertalot, *F. cassiae* Lange-Bertalot & T.Beier, *F. gondwana* Lange-Bertalot & T.Beier, *F.* cf. *magaliesmontana sensu* Beier and Lange-Bertalot [100], *F. maoriana* Lange-Bertalot & T.Beier, *F.* sp. A *sensu* Kilroy [101] - later described as *F. paulii* Kilroy & Urbánková - **Article 4**). The other recovered *Frustulia* lineages resemble two common **widespread** morphospecies, *F. crassinervia* (Brébisson) Lange-Bertalot & Krammer and *F. saxonica* Rabenhorst (formerly identified as *F. rhomboides* var. *crassinervia* and var. *saxonica*), but only one group of closely related lineages (lineages IV-VI *sensu* **Article 2**) corresponds to this distribution; the remaining *F. crassinervia-saxonica*-like lineages have been found in a single geographical area (**Europe**: *F.* cf. *maoriana sensu* **Article 5**, lineage II *sensu* **Article 2**, which was later described as *F. curvata* Kulichová & Urbánková – **Article 4**, and lineage III *sensu* **Article 2**; South Island of **New Zealand**: lineage VII *sensu* **Article 3**).

Evaluating the realized ecological niches of the *Frustulia* lineages was challenging, even though we had much more data (hundreds of strains and tens of locations) than in the *Navicula* study. Detailed morphological examination of the strains reveal lineage-specific variation, but the variability of natural populations (especially where highly variable *F. crassinervia-saxonica* morphotypes co-occurred) reduced the certainty of correct morphological identification in field samples (**Article 5**). We estimated the realized niches of some *Frustulia* lineages based on sequence abundances of individual lineages at sampling sites (**Article 2**) and using a semi-supervised classification algorithm that worked
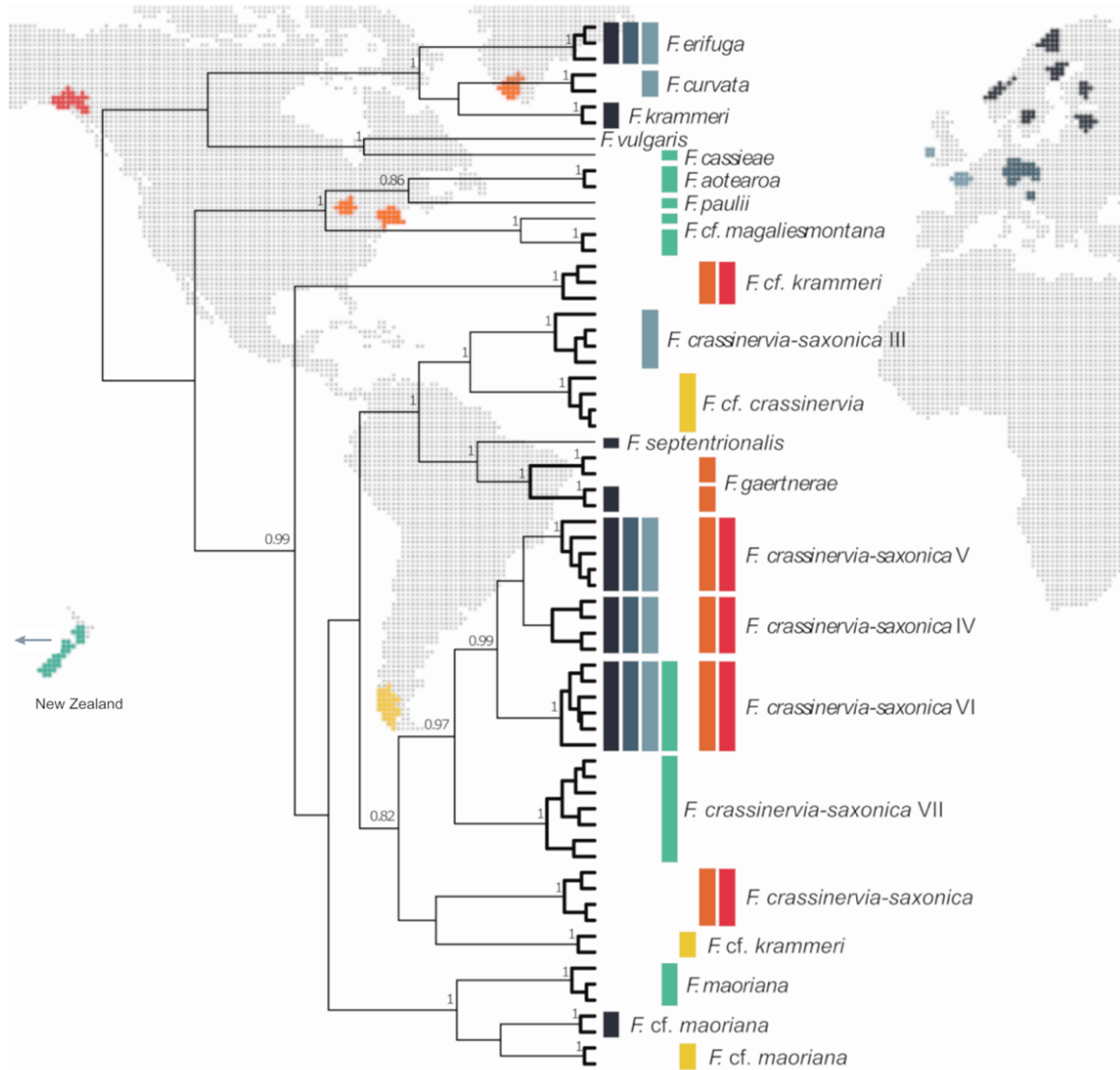
Figure 5. Phylogenetic tree based on partial LSU rDNA sequences, species identification by LM/SEM and geographical origin of *Frustulia* isolates. Explanatory notes: the Generalized Mixed Yule Coalescent model was used for tree construction and species delimitation; colors near delimited lineages correspond to areas in the background map: Europe - shades of blue (Northern Europe, Central Europe, Western Europe), green - New Zealand, yellow - Patagonia, shades of red - northern part of the western hemisphere (USA + Greenland, Canada). Adapted from a poster: Urbánková, P., J. Kulichová and V. Scharfen, *Single-locus species delimitation in the diatom genus Frustulia*, presented at the 62nd Annual Meeting of the British Phycological Society, Galway, Ireland, 2014.

with morphometric data obtained from strains examined by light (LM) and scanning electron microscopy (SEM; **Article 5**). Our data from European populations showed that i) the most frequently detected lineage VI (morphotype *F. crassinervia-saxonica*) can live in a relatively wide range of pH (3.5-6.9) and is abundant in acidic and semi-aquatic habitats; ii) lineage V (the same morphotype) is common in the littoral of circumneutral lakes; and iii) three of the Fennoscandian lineages (identified as *F. gaertnerae, F. septentrionalis, F.*

krammeri) occur within a narrow range of pH values that are not the same for the three species.

The number of lineages in our species complexes increased with increased **geographical range and habitat heterogeneity**. Despite differences in dispersal ability, this pattern was observed in both aquatic and terrestrial taxa. The largest geographical range and the highest hidden genetic diversity was achieved by sampling of semi-aquatic and terrestrial habitats where the nominal species *Pinnularia borealis* lives. The eleven lineages discovered were morphologically similar to at least four varieties of *P. borealis* (var. *borealis* Ehrenberg, var. *islandica* Krammer, var. *scalaris* (Ehrenberg) Rabenhorst [98]), but unambiguous identification of strains was not possible due to lack of clear morphological boundaries between traditional taxa (**Article 6**). Similarly to the genus *Frustulia*, more than one lineage was found within a single sample (e.g., lineages C+D+L *sensu* **Article 6**), some lineages were detected at only one sampling site (lineage A and lineage B), and some phylogenetically closely related lineages originated from distant geographical regions (e.g., Europe and islands in the Southern Ocean: lineage D and lineages F+L; Figure 6). These
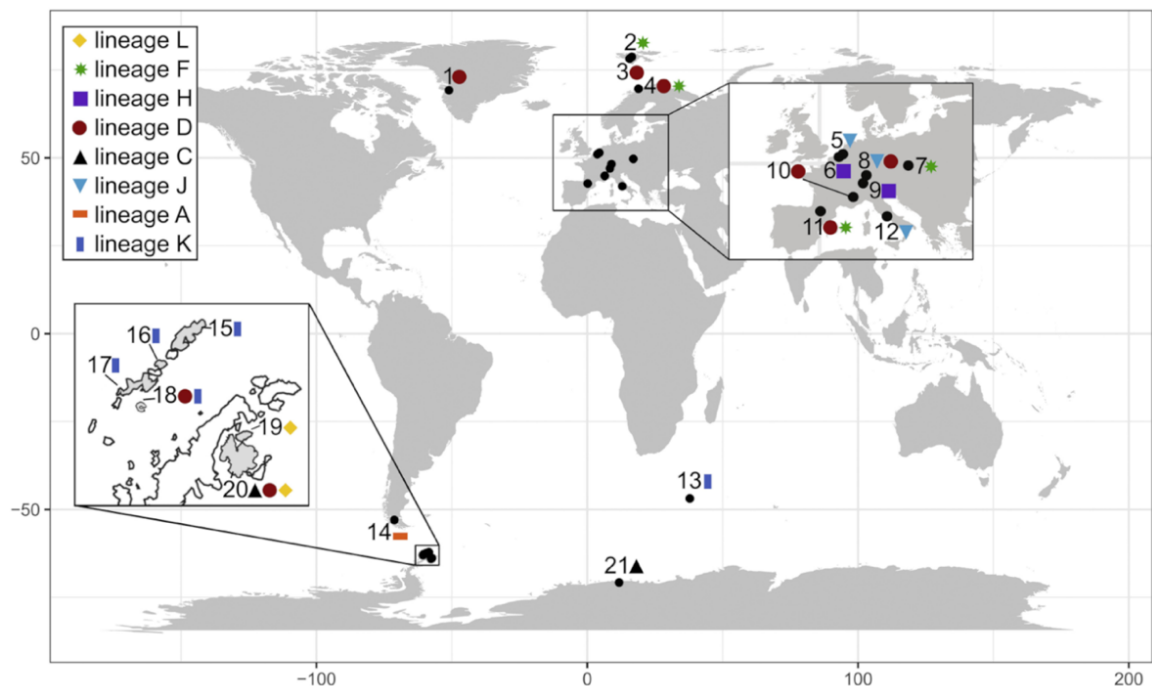
Figure 6. Geographical distribution of *Pinnularia borealis* lineages *sensu* Article 6. Explanatory notes: the lineages were delimited by maximum likelihood phylogeny (the concatenated dataset of the LSU rDNA and rbcL sequences); sampling locations are listed in Article 6, from where the figure was derived.

differences in distribution patterns among lineages indicated that they did not share the same dispersal ability and/or niche breadth. It is also likely that our molecular markers are too conservative to recognize relatively recent microevolutionary events [95].

The distribution of terrestrial and semi-aquatic diatoms is to some extent governed by the same factors that are important for aquatic diatom flora (e.g., substrate type, pH values, nutrient concentrations), but in addition they must be adapted to severe environmental conditions such as unstable water availability (humidity, precipitation, emergence of the substrate from the water), high temperature (above 40°C), freezing, and high levels of UV radiation [102, 103]. In our study of *Pinnularia borealis* (**Article 6**), we did not measure local conditions, assuming that differences in climate and habitat type (soil, wet mosses, lake littoral) were more important for diversification than, for example, physicochemical properties of water. Indeed, two studies of the *P. borealis* complex have shown that several lineages of this complex colonized aquatic habitats [95], and at least one lineage originating in Antarctica, prefer cooler temperatures than its non-Antarctic relatives [98].

Morphometric analyses of clonal strains

We examined the morphology of the problematic lineages in detail (LM, SEM, morphometrics; for our methodology see **Articles 1, 2, 6**) to find a unique combination of features for their identification. Using ultrastructural characters, we were able to distinguish two Holarctic species of the genus *Frustulia* (*F. gaertnerae*, *F. septentrionalis*) from the *F. crassinervia-saxonica* morphotypes, which are otherwise similar in size and shape (**Article 5**). **Pseudocryptic diversity** was revealed in two lineages of *N. cryptocephala* (lineage I and II): living cells have different arrangement of nuclei during cell division (**Article 1**) and their cell walls differ in the shape of the unperforated central part (Figure 7; **Article 8**; [104]).

We were unable to detect additional cases of pseudocryptic diversity despite evaluating commonly overlooked features such as the width of unperforated regions along the axial axis of cells and the appearance of organelles in living cells (**Article 6**). The results of geometric morphometrics can be considered a partial success. Some lineages could be distinguished from a statistical point of view (i.e., **semicryptic** lineages), however, without the possibility of unambiguous identification of all members belonging to a particular lineage (**Articles 2, 5, 6**). Statistical classification of cells is beneficial in studies where diatoms are mounted in permanent slides, because with the help of morphometrics, cells with unavailable DNA sequences can be assigned to semi-cryptic lineages. For taxonomic purposes, the morphology of the type material can be compared with the variability of lineages, allowing to determine which lineage is most similar to the type specimens, as was done in *N. cryptocephala* (lineage I retained the name *N. cryptocephala* and lineage II was described as a new species - *N. lothargeitlerii* [104]). Furthermore, a machine learning algorithm may use the morphology of sequenced strains to improve the automated classification of diatoms that has not been sequenced (**Article 5**). Statistical identification, however, may depend on the amount of overlap in morphological variation, as shown in

our study examining eight lineages of *Pinnularia borealis*, where classification accuracy decreased with increasing number of (semi)cryptic strains (**Article 6**).
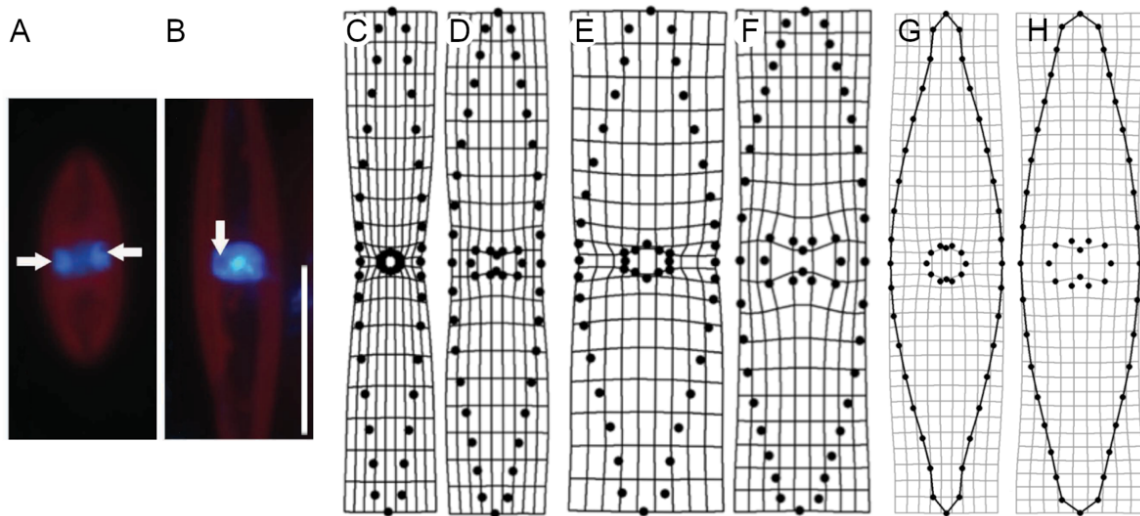

Figure 7. Pseudocryptic diversity between *Navicula cryptocephala* lineages I (A, C, E, G) and lineage II (B, D, F, H) *sensu* Article 1. **A, B**: interphase structure of the nucleus in fluorescence microscopy after DAPI staining (adapted from [104]); **C, D**: large cells of strains from Scotland (strain 460R and 27L); **E, F**: small cells of the same strains (adapted from Article 8); **G, H**: specific features of lineages I and II derived from multiple strains (adapted from [104]). Scale bar = 10 μm.

3.2. Ontogenetic and ecological signals in the morphology of diatom cell walls

Morphological changes in the life cycle

Our geometric morphometric analyses have shown that **allometry** is the most pronounced component of shape variation in diatom populations. Differences in size between the largest and smallest cells of the strains, which varied in length by tens of micrometers, erased differences between morphospecies because similar sizes clustered together regardless of species affiliation (**Article 8**). This pattern appears to be associated with shape simplification during cell size reduction - from more complex to more circular outlines (**Article 9;** Figure 2B, 7C-F). Allometry is also the most important component in clonal strains with only small size differences (**Article 6, 10**). Despite the possibility of removing size-related shape variation from the dataset (using landmark-based geometric morphometrics), this is not always advantageous. Species-specific morphology may be partially removed from the data along with the removal of allometry, as for example in the lineages of *Navicula cryptocephala*, which differ more in the shape of the apical ends and central part of the cell walls in the original dataset (**Article 8**) and less in the non-allometric dataset (**Article 1**). Analyzing cells of similar size, plotting ontogenetic trajectories of cell shape [76, 105], and analyzing ontogenetically more stable internal structures (**Article 8**; [106]; Figure 7C-H) could be possible solutions for dealing with allometry without its masking effect.

Although life cycle changes compromise the identification of diatom species, repeated inheritance and synthesis of siliceous cell walls can be used to recognize errors in morphogenesis (i.e., developmental instability) caused by growth in suboptimal conditions (reviewed in [84]). With this potential use in mind, we analyzed different genera of biradially symmetric diatoms (i.e., symmetric along apical and transapical axes, Figure 1E) that presumably grew under non-stressful conditions to assess the **disparity** among symmetrized cells and **asymmetry** within cells (see **Article 9** for our methodology).

We examined clonal strains and natural populations of diatoms to analyze variability of three asymmetric components: vertical asymmetry (causing heteropolar shape, Figure 1I), horizontal asymmetry (dorsiventral shape, Figure 1C, 1H), and transversal asymmetry (sigmoid shape). The ability to recognize the sides of cells along horizontal axis in the genus *Luticola* has allowed the calculation of fluctuating asymmetry (**Article 11**), whose variability can be related to extent of developmental instability.

Morphometric analysis over the life cycle of the three genera (*Luticola, Navicula, Sellaphora*) showed that disparity increased with decreasing size, while the relationships of size to asymmetric components (vertical, horizontal, transversal) were not consistent. Asymmetry within cells generally increased with decreasing size, but some components (not always of the same type) were weakly or insignificantly related to cell size (**Article 9**). In subsequent studies of two biradially symmetric genera, *Frustulia* and *Luticola*, it was confirmed that differences in cell sizes most likely do not have large effect on any of the components of measured asymmetry (**Article 10**) including fluctuating asymmetry (**Article 11**; [107]).

We did not expect increased disparity in small cells given their shape simplicity (increased circularity). We proposed that this relationship (together with greater asymmetry in the small cells) is the result of an accumulation of errors during morphogenesis caused by epitheca inheritance (Figure 2A) and/or suboptimal conditions inside the small frustules (**Article 9**). Our studies demonstrated that asymmetry of biradially symmetric diatoms is potentially more useful in biomonitoring studies than disparity because its variability is less sensitive to ontogenetic size changes. A second reason why asymmetry is advantageous is that its values are calculated at the level of individuals, which means that asymmetric variability is not dependent on comparison with other members of the dataset as in the case of disparity, which is higher in larger datasets and in datasets containing shape outliers (**Article 9**).

Ecologically induced phenotype

With the adoption of fine-grained taxonomy in diatoms, species identification has become much more difficult, hampering their easy use as bioindicators of recent and past changes. Diatomologists have therefore focused their interest on finding alternative metrics for assessing changes in ecological status. In addition to efforts to improve DNA-based

biomonitoring of freshwaters [108, 109], phenotypic traits above the species level (cell sizes [110, 111], shape of cell outlines [39], and life-strategies [112, 113]) have been studied with respect to environmental heterogeneity. In line with the second approach, we quantified the **shape and size of diatom communities** in a neglected type of benthic habitat - peatlands.

Peatlands can be divided into two types based on water source: minerotrophic fens fed by groundwater or surface water and ombrotrophic bogs dependent on precipitation. Water source influences acidity and the availability of inorganic nutrients (higher acidity and lower nutrient concentrations in bogs), but only partially, because other factors such as past anthropogenic acidification may have altered the hydrochemistry (for references see **Article 12**). Our results from two sampling campaigns showed that diatom sizes are significantly affected by differences in peatland types. Although the relationship between average cell sizes of whole diatom communities was insignificant along the ombro-minerotrophic gradient, pH values that significantly correlated with ombro-minerotrophy showed a non-linear correlation with average community sizes. The observed relationship showed that the sizes increased from pH 3.3 to 5.3 and then decreased with increasing pH values (up to a measured value of 7.3 - **Article 12**; Figure 8A). Aware of the pitfalls in
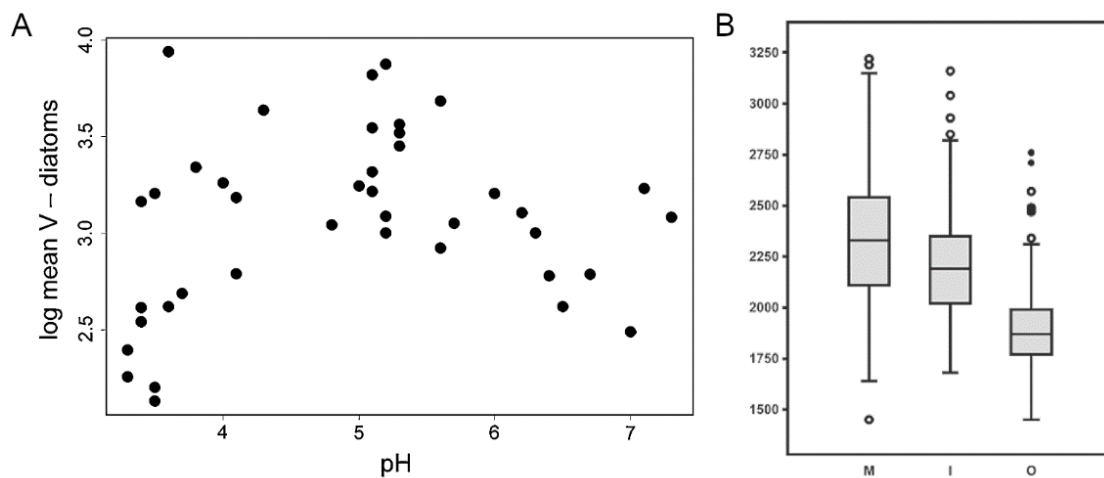

Figure 8. Cell sizes of natural populations in peatlands sampled on a gradient of ombro-minerotrophy. **A**: biovolume of whole diatom communities from European peatlands plotted against pH values (graph adapted from Article 12); **B**: centroid sizes of *Frustulia saxonica-crassinervia* populations collected in the Czech Republic from minerotrophic (M), intermediate (I), and ombrotrophic peatlands (O; graph adapted from Article 13).

comparing different datasets, I believe that our second study along a shorter pH gradient (pH from 3.6 to 5.5) and narrower taxonomic scope (analysis of cell sizes and shapes of *Frustulia* populations - **Article 13**), confirmed the predominance of smaller cells in more stressful conditions (i.e., ombrotrophic mires) and larger cells in more convenient conditions for acidophilic species (minerotrophic mires; Figure 8B). The finding of a non-

significant relationship of centroid sizes with circularity and with disparity supports the claim that the cell sizes of *Frustulia* populations reflect environmental heterogeneity rather than ontogenetic allometry. The three most likely explanations for the observed size-related patterns along our environmental gradients are optimization of surface-to-volume ratios and changes in competitiveness and/or growth rates (see **Articles 12** and **13** for details).

3.3.   Ecological and evolutionary conclusions from our results

The transition from observing organisms to explaining biological causes is not easy because similar patterns (e.g., species-area relationships, species abundance distributions, distance-decay of similarity) can emerge in different ways[12] (see [114] for details). Given the inability to establish general relationship between natural mechanisms and pattern valid across spatial and temporal scales, four fundamental processes of community ecology were proposed: **selection** (response to abiotic conditions and biotic interactions), **dispersal** (movement in space), **evolutionary diversification** (long-term changes including speciation), and **stochasticity** (an intrinsic feature of previous processes; [114, 115]). The different relative importance of these processes in natural communities is reflected in the existence of a number of ecological concepts, such as niche-assembly theories [116], neutral models [117], the metacommunity concept [118], monopolization hypothesis [119], and phylogenetic community structure [120].

The difference in the abundances of sympatric lineages depending on ecological conditions (*Frustulia crassinervia-saxonica* lineages V and VI - **Article 2**), the significant relationship between morphology and environmental gradient (cell sizes of natural communities from peatlands - **Articles 12, 13**), and the occurrence of some lineages within a narrow pH range (Fennoscandian *Frustulia* species - **Article 5**) demonstrates the relevance of the first process - selection. The effect of dispersal was historically considered negligible for common diatom morphospecies, and they were assumed to be cosmopolitan according to broadly used identification keys (e.g., [87]). Although our findings have confirmed the ability of some lineages (resembling common morphospecies) to disperse globally **(Articles 1, 3, 6)**, the distribution of many other lineages was hampered by large spatial distances (*Frustulia* lineages from Holarctic or Australasia - **Articles 4, 5, 10)**. The reduced ability to colonize new localities may also be due to low abundance of source populations as a result of slow cell division (high cost of dividing large cells of the genus *Frustulia* - **Article 10**, life of *Pinnularia borealis* in the inhospitable conditions of non-aquatic habitats - **Article 6**). Wide distribution, on the other hand, may be enabled by a broad ecological niche, rapid growth and/or tolerance to withstand desiccation (*Frustulia* lineage

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[12] finding a particular pattern does not allow for generalizations regarding the link between the process and pattern because the importance of processes depends on the spatiotemporal scales at which communities are studied

VI - **Article 5**). The third process, evolution of traits, is detectable in morphological diversification of clades that are formed by both morphologically similar and divergent lineages (**Articles 1, 10**) and in the transition to a different habitat type (from terrestrial habitats to freshwater littoral - **Article 6**). Further examples from other diatom groups can be found in our recent review (**Article 7**).

Consistent with de Queiroz's view on speciation [50], the lineages of our species complexes have evolved in their own way, with the aforementioned processes influencing their genes, morphology, ecology, and distribution various ways throughout the evolution of metapopulations and community assembly.

4. REFERENCES

1. Round, F.E., R.M. Crawford, and D.G. Mann, *The Diatoms. Biology and Morphology of the Genera*. **1990**: Cambridge University Press. 747 pp.

2. Mann, D.G., R.M. Crawford, and F.E. Round, *Bacillariophyta*, in *Handbook of the Protists*, J.M. Archibald, A.G.B. Simpson, and C.H. Slamovits, Editors. **2017**, Springer International Publishing. p. 205-266.

3. Wang, J., S. Cao, C. Du, and D. Chen, *Underwater locomotion strategy by a benthic pennate diatom Navicula sp.* Protoplasma, **2013**. 250: p. 1203-1212.

4. Benoiston, A.-S., F.M. Ibarbalz, L. Bittner, L. Guidi, O. Jahn, S. Dutkiewicz, and C. Bowler, *The evolution of diatoms and their biogeochemical functions.* Philosophical Transactions of the Royal Society B: Biological Sciences, **2017**. 372: 20160397.

5. Tréguer, P., C. Bowler, B. Moriceau, S. Dutkiewicz, M. Gehlen, O. Aumont, L. Bittner, R. Dugdale, Z. Finkel, D. Iudicone, O. Jahn, L. Guidi, M. Lasbleiz, K. Leblanc, M. Levy, and P. Pondaven, *Influence of diatom diversity on the ocean biological carbon pump.* Nature Geoscience, **2018**. 11(1): p. 27-37.

6. Brown, J.W. and U. Sorhannus, *A molecular genetic timescale for the diversification of autotrophic stramenopiles (Ochrophyta): substantive underestimation of putative fossil ages.* PLoS One, **2010**. 5(9): e12759.

7. Guillou, L., M.J. Chrétiennot-Dinet, L.K. Medlin, H. Claustre, S.L.d. Goër, and D. Vaulot, *Bolidomonas: a new genus with two species belonging to a new algal class, the Bolidophyceae (Heterokonta).* Journal of Phycology, **1999**. 35(2): p. 368-381.

8. Edlund, M.B. and E.F. Stoermer, *Ecological, evolutionary, and systematic significance of diatom life histories.* Journal of Phycology, **1997**. 33(6): p. 897-918.

9. Booth, B.C. and H.J. Marchant, *Parmales, a new order of marine Chrysophytes, with descriptions of three new genera and seven new species.* Journal of Phycology, **1987**. 23(s2): p. 245-260.

10. Ichinomiya, M., A.L. Dos Santos, P. Gourvil, S. Yoshikawa, M. Kamiya, K. Ohki, S. Audic, C. De Vargas, M.-H. Noël, and D. Vaulot, *Diversity and oceanic distribution of the Parmales (Bolidophyceae), a picoplanktonic group closely related to diatoms.* The ISME Journal, **2016**. 10(10): p. 2419-2434.

11. Nakov, T., J.M. Beaulieu, and A.J. Alverson, *Diatoms diversify and turn over faster in freshwater than marine environments.* Evolution, **2019**. 73(12): p. 2497-2511.

12. Kooistra, W.H., R. Gersonde, L.K. Medlin, and D.G. Mann, *The origin and evolution of the diatoms: their adaptation to a planktonic existence*, in *Evolution of Primary Producers in the Sea*, P.G. Falkowskii and A.H. Knol Editors. **2007**, Academic Press. p. 207-249.

13. Alverson, A.J., J.J. Cannone, R.R. Gutell, and E.C. Theriot, *The evolution of elongate shape in diatoms.* Journal of Phycology, **2006**. 42(3): p. 655-668.

14. Nakov, T., J.M. Beaulieu, and A.J. Alverson, *Insights into global planktonic diatom diversity: The importance of comparisons between phylogenetically equivalent units that account for time.* The ISME Journal, **2018**. 12(11): p. 2807-2810.

15. Ashworth, M.P., T. Nakov, and E.C. Theriot, *Revisiting Ross and Sims (1971): toward a molecular phylogeny of the Biddulphiaceae and Eupodiscaceae (Bacillariophyceae).* Journal of Phycology, **2013**. 49(6): p. 1207-1222.

16. De Luca, D., W.H. Kooistra, D. Sarno, C.C. Gaonkar, and R. Piredda, *Global distribution and diversity of Chaetoceros (Bacillariophyta, Mediophyceae): integration of classical and novel strategies.* PeerJ, **2019**. 7: e7410.

17. Medlin, L.K., *Evolution of the diatoms: major steps in their evolution and a review of the supporting molecular and morphological evidence.* Phycologia, **2016**. 55(1): p. 79-103.

18. Evans, K.M., A.H. Wortley, G.E. Simpson, V.A. Chepurnov, and D.G. Mann, *A molecular systematic approach to explore diversity within the Sellaphora pupula species complex (Bacillariophyta).* Journal of Phycology, **2008**. 44(1): p. 215-231.

19. Rovira, L., R. Trobajo, S. Sato, C. Ibáñez, and D.G. Mann, *Genetic and physiological diversity in the diatom Nitzschia inconspicua.* Journal of Eukaryotic Microbiology, **2015**. 62(6): p. 815-832.

20. Vanormelingen, P., K.M. Evans, V. Chepurnov, W. Vyverman, and D.G. Mann, *Molecular species discovery in the diatom Sellaphora and its congruence with mating trials.* Fottea, **2013**. 13(2): p. 133-148.

21. Cerino, F., L. Orsini, D. Sarno, C. Dell'Aversano, L. Tartaglione, and A. Zingone, *The alternation of different morphotypes in the seasonal cycle of the toxic diatom Pseudo-nitzschia galaxiae.* Harmful Algae, **2005**. 4(1): p. 33-48.

22. Nakov, T., E.C. Ruck, Y. Galachyants, S.A. Spaulding, and E.C. Theriot, *Molecular phylogeny of the Cymbellales (Bacillariophyceae, Heterokontophyta) with a comparison of models for accommodating rate variation across sites.* Phycologia, **2014**. 53(4): p. 359-373.

23. Mann, D.G., R. Trobajo, S. Sato, C. Li, A. Witkowski, F. Rimet, M.P. Ashworth, R.M. Hollands, and E.C. Theriot, *Ripe for reassessment: A synthesis of available molecular data for the speciose diatom family Bacillariaceae.* Molecular Phylogenetics and Evolution, **2021**. 158: 106985.

24. Ruck, E.C. and E.C. Theriot, *Origin and evolution of the canal raphe system in diatoms.* Protist, **2011**. 162(5): p. 723-737.

25. Ashworth, M.P., C.S. Lobban, A. Witkowski, E.C. Theriot, M.J. Sabir, M.N. Baeshen, N.H. Hajarah, N.A. Baeshen, J.S. Sabir, and R.K. Jansen, *Molecular and morphological investigations of the stauros-bearing, raphid pennate diatoms (Bacillariophyceae): Craspedostauros EJ Cox, and Staurotropis TBB Paddock, and their relationship to the rest of the Mastogloiales.* Protist, **2017**. 168(1): p. 48-70.

26. Cox, E.J., *Ontogeny, homology, and terminology - wall morphogenesis as an aid to character recognition and character state definition for pennate diatom systematics.* Journal of Phycology, **2012**. 48(1): p. 1-31.

27. Mann, D., *The origins of shape and form in diatoms: the interplay between morphometric studies and systematics*, in *Shape and Form in Plants and Fungi*, D.S. Ingram and A. Hudson, Editors. **1994**, Academic Press. p. 17-38.

28. Gargas, C.B., E.C. Theriot, M.P. Ashworth, and J.R. Johansen, *Phylogenetic analysis reveals that the 'Radial Centric' diatom Orthoseira Thwaites (Orthoseiraceae, Bacillariophyta) is a member of a 'Multipolar' diatom lineage.* Protist, **2018**. 169(6): p. 803-825.

29. Kaczmarska, I. and J.M. Ehrman, *Enlarge or die! An auxospore perspective on diatom diversification.* Organisms Diversity and Evolution, **2021**. 21(1): p. 1-23.

30. Geitler, L., *Der Formwechsel der pennaten Diatomeen (Kieselalgen).* Archiv für Protistenkunde, **1932**. 78: p. 1-226.

31. Jewson, D., *Size reduction, reproductive strategy and the life cycle of a centric diatom.* Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, **1992**. 336(1277): p. 191-213.

32. Mann, D.G., *Why didn't Lund see sex in Asterionella? A discussion of the diatom life cycle in nature.* Algae and the Aquatic Environment, **1988**. 29: p. 385-412.

33. D'Alelio, D., M.R. d'Alcalà, L. Dubroca, , Diana Sarn, A. Zingone, and M. Montresor, *The time for sex: A biennial life cycle in a marine planktonic diatom.* Limnology and Oceanography, **2010**. 55(1): p. 106-114.

34. Mann, D.G., *Discovering diatom species: is a long history of disagreements about species-level taxonomy now at an end?* Plant Ecology and Evolution, **2010**. 143(3): p. 251-264.

35. Levkov, Z., D.M. Williams, D. Nikolovska, S. Tofilovska, and Z. Cakar, *The use of diatoms in forensic science: advantages and limitations of the diatom test in cases of drowning*, in *The Archaeological and Forensic Applications of Microfossils: A Deeper Understanding of Human History.*, M. Williams, T. Hill, I. Boomer, and I.P. Wilkinson, Editors. **2017**, Geological Society of London. p. 261-277.

36. Bozarth, A., U.G. Maier, and S. Zauner, *Diatoms in biotechnology: modern tools and applications.* Applied Microbiology and Biotechnology, **2009**. 82(2): p. 195-201.

37. Wang, Y., J. Cai, Y. Jiang, X. Jiang, and D. Zhang, *Preparation of biosilica structures from frustules of diatoms and their applications: current state and perspectives.* Applied Microbiology and Biotechnology, **2013**. 97(2): p. 453-460.

38. Mann, D.G., *The species concept in diatoms.* Phycologia, **1999**. 38(6): p. 437-495.

39. Potapova, M. and P.B. Hamilton, *Morphological and ecological variation within the Achnanthidium minutissimum (Bacillariophyceae) species complex.* Journal of Phycology, **2007**. 43(3): p. 561-575.

40. Kooistra, W.H., M. De Stefano, D.G. Mann, N. Salma, and L.K. Medlin, *Phylogenetic position of Toxarium, a pennate-like lineage within centric diatoms (Bacillariophyceae).* Journal of Phycology, **2003**. 39(1): p. 185-197.

41. Rose, D.T. and E.J. Cox, *What constitutes Gomphonema parvulum? Long-term culture studies show that some varieties of G. parvulum belong with other Gomphonema species.* Plant Ecology and Evolution, **2014**. 147(3): p. 366-373.

42. Vanelslander, B., V. Créach, P. Vanormelingen, A. Ernst, V.A. Chepurnov, E. Sahan, G. Muyzer, L.J. Stal, W. Vyverman, and K. Sabbe, *Ecological differentiation between sympatric pseudocryptic species in the estuarine benthic diatom Navicula phyllepta (Bacillariophyceae).* Journal of Phycology, **2009**. 45(6): p. 1278-1289.

43. Degerlund, M., S. Huseby, A. Zingone, D. Sarno, and B. Landfald, *Functional diversity in cryptic species of Chaetoceros socialis Lauder (Bacillariophyceae).* Journal of Plankton Research, **2012**. 34(5): p. 416-431.

44. Mann, D.G. and K.M. Evans, *Molecular genetics and the neglected art of diatomics*, in *Unravelling the algae: the past, present, and future of algal systematics*, J. Brodie and J. Lewis, Editors. **2007**, CRC Press. p. 231-265.

45. Vanormelingen, P., V.A. Chepurnov, D.G. Mann, K. Sabbe, and W. Vyverman, *Genetic divergence and reproductive barriers among morphologically heterogeneous sympatric clones of Eunotia bilunaris sensu lato (Bacillariophyta).* Protist, **2008**. 159(1): p. 73-90.

46. Kaczmarska, I., L. Mather, I.A. Luddington, F. Muise, and J.M. Ehrman, *Cryptic diversity in a cosmopolitan diatom known as Asterionellopsis glacialis (Fragilariaceae): Implications for ecology, biogeography, and taxonomy.* American Journal of Botany, **2014**. 101(2): p. 267-286.

47. Mann, D.G., S. Sato, R. Trobajo, P. Vanormelingen, and C. Souffreau, *DNA barcoding for species identification and discovery in diatoms.* Cryptogamie, Algologie, **2010**. 31(4): p. 557-577.

48. Moniz, M.B. and I. Kaczmarska, *Barcoding diatoms: Is there a good marker?* Molecular Ecology Resources, **2009**. 9(s1): p. 65-74.

49. Hamsher, S.E., K.M. Evans, D.G. Mann, A. Poulickova, and G.W. Saunders, *Barcoding diatoms: exploring alternatives to COI-5P.* Protist, **2011**. 162(3): p. 405-422.

50. de Queiroz, K., *Ernst Mayr and the modern concept of species.* Proceedings of the National Academy of Sciences of the United States of America, **2005**. 102(s1): p. 6600-6607.

51. Amato, A., W.H. Kooistra, J.H. Ghiron, D.G. Mann, T. Proschold, and M. Montresor, *Reproductive isolation among sympatric cryptic species in marine diatoms.* Protist, **2007**. 158(2): p. 193-207.

52. Trobajo, R., E. Clavero, V.A. Chepurnov, K. Sabbe, D.G. Mann, S. Ishihara, and E.J. Cox, *Morphological, genetic and mating diversity within the widespread bioindicator Nitzschia palea (Bacillariophyceae).* Phycologia, **2009**. 48(6): p. 443-459.

53. Mann, D.G. and S.J.M. Droop, *Biodiversity, biogeography and conservation of diatoms*, in *Biogeography of Freshwater Algae*. **1996**, Kluwer Academic Publishers. p. 19-32.

54. Mann, D.G. and P. Vanormelingen, *An inordinate fondness? The number, distributions, and origins of diatom species.* Journal of Eukaryotic Microbiology, **2013**. 60(4): p. 414–420.

55. De Clerck, O., M.D. Guiry, F. Leliaert, Y. Samyn, and H. Verbruggen, *Algal taxonomy: a road to nowhere?* Journal of Phycology, **2013**. 49(2): p. 215-225.

56. Alverson, A.J., *Molecular systematics and the diatom species.* Protist, **2008**. 159(3): p. 339-53.

57. Rimet, F., R. Trobajo, D.G. Mann, L. Kermarrec, A. Franc, I. Domaizon, and A. Bouchez, *When is sampling complete? The effects of geographical range and marker choice on perceived diversity in Nitzschia palea (Bacillariophyta).* Protist, **2014**. 165(3): p. 245-59.

58. Abarca, N., R. Jahn, J. Zimmermann, and N. Enke, *Does the cosmopolitan diatom Gomphonema parvulum (Kutzing) Kutzing have a biogeography?* PLoS One, **2014**. 9(1): e86885.

59. Pérez-Burillo, J., R. Trobajo, M. Leira, F. Keck, F. Rimet, J. Sigró, and D.G. Mann, *DNA metabarcoding reveals differences in distribution patterns and ecological preferences among genetic variants within some key freshwater diatom species.* Science of the Total Environment, **2021**. 798: 149029.

60. De Luca, D., R. Piredda, D. Sarno, and W.H. Kooistra, *Resolving cryptic species complexes in marine protists: phylogenetic haplotype networks meet global DNA metabarcoding datasets.* The ISME Journal, **2021**. 15(7): p. 1931-1942.

61. Lakeman, M.B., P. von Dassow, and R.A. Cattolico, *The strain concept in phytoplankton ecology.* Harmful Algae, **2009**. 8(5): p. 746-758.

62. Rimet, F., E. Gusev, M. Kahlert, M.G. Kelly, M. Kulikovskiy, Y. Maltsev, D.G. Mann, M. Pfannkuchen, R. Trobajo, and V. Vasselon, *Diat. barcode, an open-access curated barcode library for diatoms.* Scientific Reports, **2019**. 9(1): p. 1-12.

63. Clark, K., I. Karsch-Mizrachi, D.J. Lipman, J. Ostell, and E.W. Sayers, *GenBank.* Nucleic Acids Research, **2016**. 44(D1): p. D67-72.

64. Casteleyn, G., V.A. Chepurnov, F. Leliaert, D.G. Mann, S.S. Bates, N. Lundholm, L. Rhodes, K. Sabbe, and W. Vyverman, *Pseudo-nitzschia pungens (Bacillariophyceae): a cosmopolitan diatom species?* Harmful Algae, **2008**. 7(2): p. 241-257.

65. Kooistra, W.H., D. Sarno, S. Balzano, H. Gu, R.A. Andersen, and A. Zingone, *Global diversity and biogeography of Skeletonema species (Bacillariophyta).* Protist, **2008**. 159(2): p. 177-193.

66. Poulíčková, A., J. Špačková, M.G. Kelly, M. Duchoslav, and D.G. Mann, *Ecological variation within Sellaphora species complexes (Bacillaryophyceae): specialists or generalists?* Hydrobiologia, **2008**. 614: p. 373-386.

67. Adams, D.C., F.J. Rohlf, and D.E. Slice, *Geometric morphometrics: Ten years of progress following the 'revolution'.* Italian Journal of Zoology, **2004**. 71(1): p. 5-16.

68. Rohlf, F.J. and L.F. Marcus, *A revolution in morphometrics.* Trends in Ecology and Evolution, **1993**. 8(4): p. 129-132.

69. Pappas, J.L., J.P. Kociolek, and E.F. Stoermer, *Quantitative morphometric methods in diatom research.* Nova Hedwigia, Beiheft, **2014**. 143: p. 281-306.

70. Du Buf, H. and M.M. Bayer, eds. *Automatic Diatom Identification*. Series in Machine Perception and Artificial Intelligence. Vol. 51. **2002**, World Scientific. 316 pp.

71. Kloster, M., G. Kauer, and B. Beszteri, *SHERPA: an image segmentation and outline feature extraction tool for diatoms and other objects.* BMC bioinformatics, **2014**. 15(1): 218.

72. Wishkerman, A. and P.B. Hamilton, *DiaCurv: a value-based curvature analysis application in diatom taxonomy.* Diatom research, **2017**. 32(3): p. 351-358.

73. Savriama, Y., J. Neustupa, and C.P. Klingenberg, *Geometric morphometrics of symmetry and allometry in Micrasterias rotata (Zygnemophyceae, Viridiplantae).* Nova Hedwigia, Beiheft, **2010**. 136: p. 43-54.

74. Mitteroecker, P. and P. Gunz, *Advances in Geometric Morphometrics.* Evolutionary Biology, **2009**. 36(2): p. 235-247.

75. Klingenberg, C.P., *Evolution and development of shape: integrating quantitative approaches.* Nature Reviews Genetics, **2010**. 11(9): p. 623-635.

76. English, J.D. and M.G. Potapova, *Ontogenetic and interspecific valve shape variation in the Pinnatae group of the genus Surirella and the description of S. lacrimula sp. nov.* Diatom Research, **2012**. 27(1): p. 9-27.

77. Foote, M., *Contributions of individual taxa to overall morphological disparity.* Paleobiology, **1993**. 19(4): p. 403-419.

78. Roy, K. and M. Foote, *Morphological approaches to measuring biodiversity.* Trends in Ecology and Evolution, **1997**. 12(7): p. 277-281.

79. Neustupa, J., K. Černá, and J. Šťastný, *Diversity and morphological disparity of desmid assemblages in Central European peatlands.* Hydrobiologia, **2009**. 630(1): p. 243-256.

80. Savriama, Y., *A step-by-step guide for geometric morphometrics of floral symmetry.* Frontiers in Plant Science, **2018**. 9: 1433.

81. Graham, J.H., J.M. Emlen, D.C. Freeman, L.J. Leamy, and J.A. Kieser, *Directional asymmetry and the measurement of developmental instability.* Biological Journal of the Linnean Society, **1998**. 64(1): p. 1-16.

82. Mann, D.G., *Symmetry and cell division in raphid diatoms.* Annals of Botany, **1983**. 52(4): p. 573-581.

83. Klingenberg, C.P., *Analyzing fluctuating asymmetry with geometric morphometrics: concepts, methods, and applications.* Symmetry, **2015**. 7(2): p. 843-934.

84. Lavoie, I., P.B. Hamilton, S. Morin, S.K. Tiam, M. Kahlert, S. Gonçalves, E. Falasco, C. Fortin, B. Gontero, and D. Heudre, *Diatom teratologies as biomarkers of contamination: Are all deformities ecologically meaningful?* Ecological Indicators, **2017**. 82: p. 539-550.

85. Nakov, T., J.M. Beaulieu, and A.J. Alverson, *Accelerated diversification is related to life history and locomotion in a hyperdiverse lineage of microbial eukaryotes (Diatoms, Bacillariophyta).* New Phytologist, **2018**. 219(1): p. 462-473.

86. Poulíčková, A. and D.G. Mann, *Sexual reproduction in Navicula cryptocephala (Bacillariophyceae).* Journal of Phycology, **2006**. 42(4): p. 872-886.

87. Krammer, K. and H. Lange-Bertalot, *Süwasserflora von Mitteleuropa, Vol. 2(1)*, ed. H. Ettl, J. Gerloff, H. Heynig, and D. Mollenhauer. **1986**: G. Fischer. 876 pp.

88. Lange-Bertalot, H., *Navicula sensu stricto. 10 genera separated from Navicula sensu lato. Frustulia*, in *Diatoms of Europe - Diatoms of European Inland Waters and Comparable Habitats.*, H. Lange-Bertalot, Editor. **2001**, A.R.G. Gantner Verlag K.G. p. 1-526.

89. Lange-Bertalot, H. and R. Jahn, *On the identity of Navicula (Frustulia) rhomboides and Frustulia saxonica (Bacillariophyceae).* Systematics and Geography of Plants, **2000**. 70(2): p. 255-261.

90. Mann, D.G., *Freshwater algae: Taxonomy, biogeography and conservation - Book Review.* Phycologia, **2001**. 40(4): p. 387-389.

91. Siver, P.A. and G. Baskette, *A morphological examination of Frustulia (Bacillariophyceae) from the Ocala National Forest, Florida, USA.* Canadian Journal of Botany, **2004**. 82(5): p. 629-644.

92. Bouchard, A.J., P.B. Hamilton, A.M. Savoie, and J.R. Starr, *Molecular and morphological data reveal hidden diversity in common North American Frustulia species (Amphipleuraceae).* Diatom Research, **2019**. 34(4): p. 205-223.

93. Pinseel, E., P. Vanormelingen, P.B. Hamilton, W. Vyverman, B. Van de Vijver, and K. Kopalova, *Molecular and morphological characterization of the Achnanthidium minutissimum complex (Bacillariophyta) in Petuniabukta (Spitsbergen, High Arctic) including the description of A. digitatum sp. nov.* European Journal of Phycology, **2017**. 52(3): p. 264-280.

94. Kollár, J., E. Pinseel, W. Vyverman, and A. Poulickova, *A time-calibrated multi-gene phylogeny provides insights into the evolution, taxonomy and DNA barcoding of the Pinnularia gibba group (Bacillariophyta).* Fottea, **2021**. 21(1): p. 62-72.

95. Pinseel, E., S.B. Janssens, E. Verleyen, P. Vanormelingen, T.J. Kohler, E.M. Biersma, K. Sabbe, B. Van de Vijver, and W. Vyverman, *Global radiation in a rare biosphere soil diatom.* Nature Communications, **2020**. 11(1): p. 1-12.

96. Evans, K.M., V.A. Chepurnov, H.J. Sluiman, S.J. Thomas, B.M. Spears, and D.G. Mann, *Highly differentiated populations of the freshwater diatom Sellaphora capitata suggest limited dispersal and opportunities for allopatric speciation.* Protist, **2009**. 160(3): p. 386-396.

97. Vanormelingen, P., K.M. Evans, D.G. Mann, S. Lance, A.E. Debeer, S. D'hondt, T. Verstraete, L. De Meester, and W. Vyverman, *Genotypic diversity and differentiation among populations of two benthic freshwater diatoms as revealed by microsatellites.* Molecular Ecology, **2015**. 24(17): p. 4433-4448.

98. Souffreau, C., P. Vanormelingen, B. Van de Vijver, T. Isheva, E. Verleyen, K. Sabbe, and W. Vyverman, *Molecular evidence for distinct Antarctic lineages in the cosmopolitan terrestrial diatoms Pinnularia borealis and Hantzschia amphioxys.* Protist, **2013**. 164(1): p. 101-115.

99. Créach, V., A. Ernst, K. Sabbe, B. Vanelslander, W. Vyverman, and L.J. Stal, *Using quantitative PCR to determine the distribution of a semicryptic benthic diatom, Navicula phyllepta (Bacillariophyceae).* Journal of Phycology, **2006**. 42(5): p. 1142-1154.

100. Beier, T. and H. Lange-Bertalot, *A synopsis of cosmopolitan, rare and new Frustulia species (Bacillariophyceae) from ombrotrophic peat bogs and minerotrophic swamps in New Zealand.* Nova Hedwigia, **2007**. 85(1): p. 73-91.

101. Kilroy, C., *Diatom communities in New Zealand subalpine mire pools: distribution, ecology and taxonomy of endemic and cosmopolitan taxa*, in *School of Biological Sciences*. 2007, University of Canterbury. p. 1-227.

102. Johansen, J.R., *Diatoms of aerial habitats*, in *The Diatoms: Applications for the Environmental and Earth Sciences*, E.F. Stoermer and J.P. Smol, Editors. **2010**, Cambridge University Press. p. 264-274.

103. Souffreau, C., P. Vanormelingen, E. Verleyen, K. Sabbe, and W. Vyverman, *Tolerance of benthic diatoms from temperate aquatic and terrestrial habitats to experimental desiccation and temperature stress.* Phycologia, **2010**. 49(4): p. 309-324.

104. Poulíčková, A., J. Neustupa, P. Hašler, O. Tomanec, and E.J. Cox, *A new species, Navicula lothargeitleri sp. nov., within the Navicula cryptocephala complex (Bacillariophyceae).* Phytotaxa, **2016**. 273(1): p. 23-33.

105. Woodard, K. and J. Neustupa, *Geometric morphometrics of bilateral asymmetry in Eunotia bilunaris (Eunotiales, Bacillariophyceae) as a tool for the quantitative assessment of teratogenic deviations in frustule shapes.* Symmetry, **2022**. 14(1): 42.

106. Kermarrec, L., A. Bouchez, F. Rimet, and J.-F. Humbert, *First evidence of the existence of semi-cryptic species and of a phylogeographic structure in the Gomphonema parvulum (Kützing) Kützing complex (Bacillariophyta).* Protist, **2013**. 164(5): p. 686-705.

107. Woodard, K. and J. Neustupa, *Morphometric asymmetry of frustule outlines in the pennate diatom Luticola poulickovae (Bacillariophyceae).* Symmetry, **2016**. 8(12): 150.

108. Tapolczai, K., F. Keck, A. Bouchez, F. Rimet, M. Kahlert, and V. Vasselon, *Diatom DNA metabarcoding for biomonitoring: strategies to avoid major taxonomical and bioinformatical biases limiting molecular indices capacities.* Frontiers in Ecology and Evolution, **2019**. 7: 409.

109. Vasselon, V., A. Bouchez, F. Rimet, S. Jacquet, R. Trobajo, M. Corniquel, K. Tapolczai, and I. Domaizon, *Avoiding quantification bias in metabarcoding: Application of a cell biovolume correction factor in diatom molecular biomonitoring.* Methods in Ecology and Evolution, **2018**. 9(4): p. 1060-1069.

110. Lavoie, I., J. Lento, and A. Morin, *Inadequacy of size distributions of stream benthic diatoms for environmental monitoring.* Journal of the North American Benthological Society, **2010**. 29(2): p. 586-601.

111. Snoeijs, P., B. Svenja, and M. Potapova, *The importance of diatom cell size in community analysis.* Journal of Phycology, **2002**. 38(2): p. 265-272.

112. Berthon, V., A. Bouchez, and F. Rimet, *Using diatom life-forms and ecological guilds to assess organic pollution and trophic level in rivers: a case study of rivers in south-eastern France.* Hydrobiologia, **2011**. 673(1): p. 259-271.

113. Tapolczai, K., A. Bouchez, C. Stenger-Kovács, J. Padisák, and F. Rimet, *Trait-based ecological classifications for benthic algae: review and perspectives.* Hydrobiologia, **2016**. 776(1): p. 1-17.

114. Vellend, M., *Conceptual synthesis in community ecology.* Quarterly Review of Biology, **2010**. 85(2): p. 183-206.

115. Thompson, P.L., L.M. Guzman, L. De Meester, Z. Horváth, R. Ptacnik, B. Vanschoenwinkel, D.S. Viana, and J.M. Chase, *A process-based metacommunity framework linking local and regional scale community ecology.* Ecology Letters, **2020**. 23(9): p. 1314-1329.

116. Pocheville, A., *The ecological niche: history and recent controversies*, in *Handbook of Evolutionary Thinking in the Sciences*, T. Heams, P. Huneman, G. Lecointre, and M. Silberstein, Editors. **2015**, Springer. p. 547-586.

117. Rosindell, J., S.P. Hubbell, and R. Etienne, *The unified neutral theory of biodiversity and biogeography at age ten.* Trends in Ecology and Evolution, **2011**. 26(7): p. 340-348.

118. Leibold, M.A., M. Holyoak, N. Mouquet, P. Amarasekare, J.M. Chase, M.F. Hoopes, R.D. Holt, J.B. Shurin, R. Law, D. Tilman, M. Loreau, and A. Gonzalez, *The metacommunity concept: a framework for multi-scale community ecology.* Ecology Letters, **2004**. 7(7): p. 601-613.

119. De Meester, L., A. Gómez, B. Okamura, and K. Schwenk, *The Monopolization Hypothesis and the dispersal - gene flow paradox in aquatic organisms.* Acta Oecologica, **2002**. 23(3): p. 121-135.

120. Webb, C.O., D.D. Ackerly, M.A. McPeek, and M.J. Donoghue, *Phylogenies and community ecology.* Annual Review of Ecology and Systematics, **2002**. 33(1): p. 475-505.