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DIVERSITY AND DISTRIBUTION OF EPIPHYTIC DIATOMS

Ph.D. Thesis

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DECLARATION

I declare that I wrote this Ph.D. only by myself. All the sources used are included in References. The published results have been approved with the help of mentioned co-authors.

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ABSTRACT

This work is focused on one group of sessile diatoms that live attached to water plants – epiphytic diatoms. Many aspects of their life are unique and have not been explored enough yet, even though they may create considerable parts of primary production, and despite their importance in biomonitoring and paleolimnological reconstructions. The questions of substrate specificity, epiphytic diatom diversity, the range of species complexes within epiphytic diatom communities and the methods used for evaluation of the ecological status were investigated.

The study of epiphytic diatoms was based on the diatom sampling and measuring the ecological parameters in the field, preparation of the permanent diatom slides, analysis of the species composition of the diatom communities and the statistical evaluation of acquired data. The aim of the work was to find the answers to the following question: i) Does the epiphytic diatom composition reflect the ecological parameters of the locality? ii) Does the type of macrophyte sampled influence the community composition? iii) What is the overall diversity of the epiphytic diatom communities? iv) What is the composition and frequency of the problematic species complexes? v) Do the unresolved species complexes reflect the state of water less precisely than other species do? vi) Are there any species complexes that if resolved could improve biomonitoring?

Epiphytic diatoms reflected the ecological parameters of water in all investgated localities. The host-substrate preference was negligible in waters of higher trophy, in the lower trophy some significant specificity was found. Epiphytic assemblages have high overall diversity with considerable proportion of species complexes consisting of cryptic or semi-cryptic species. Although the pure genus level identification seemed appropriate for the routine monitoring, fine taxonomic resolution still shows sufficient variance related to the

environmental variable and increases the sharpness of classification. Species complexes showed lower sensitivity to change in phosphorus concentration than the rest of species. Some cryptic species have potential to improve bio-assessment models. Therefore resolving the species complexes is a crucial and urgent issue that would result also in better understanding the ecological state of waters.

ABSTRAKT (ČESKY)

Tato disertační práce je zaměřena na jednu skupinu bentických rozsivek – na epifytické rozsivky, tj. na ty, které žijí přisedle na rostlinném substrátu. Mnoho aspektů jejich života není totiž stále dostatečně objasněno, přestože mohou tvořit velmi podstatnou část primární produkce a navzdory jejich důležitosti v biomonitoringu a paleolimnologických rekonstrukcích. Byla zkoumána substrátová specifita, diverzita epifytických rozsivek, rozsah druhových komplexů ve společenstvu epifytických rozsivek a také metody používané pro hodnocení ekologického stavu vod.

Tato studie byla založena na vzorkování, měření ekologických parametrů v terénu, na přípravě trvalých rozsivkových preparátů, analýze druhového složení rozsivkových společenstev a na statistickém zpracování získaných dat. Cílem práce bylo najít odpovědi na následující otázky: i) Reflektuje složení epifytických rozsivek ekologické parametry lokality? ii) Ovlivňuje typ vodní rostliny složení společenstva epifytických rozsivek? iii) Jaká je celková diverzita společenstva epifytických rozsivek? iv) Jak velká část společenstva je tvořena problematickými druhovými komplexy, a jaké je jejich složení? v) Reflektují nerozlišené druhové komplexy stav vody méně přesně než jiné druhy? vi) Existují druhové komplexy, jejichž rozlišení by mohlo zkvalitnit biomonitoring?

Epifytické rozsivky odpovídaly na ekologické parametry vody ve všech zkoumaných lokalitách. Substrátová specifita byla zanedbatelná ve vodách vyšší trofie, v čistém horském jezeře Valagola byla nalezena signifikantní substrátová specifita. Skupina epifytických rozsivek má celkově velkou druhovou bohatost a podstatná část je tvořena druhovými komplexy, tvořenými kryptickými a semikryptickými druhy. Ačkoliv se pouhé rodové určení zdá být naprosto dostačující pro rutinní monitoring, jemné taxonomické rozlišení stále vykazuje odchylku v souvislosti s proměnnými prostředí a zvyšuje přesnost klasifikace.

Nerozlišené druhové komplexy měly nízkou citlivost pro změny v koncentraci fosforu. Některé kryptické druhy mají potenciál zlepšit bioindikační modely. Proto rozlišení těchto druhových komplexů je zásadní a naléhavou záležitostí, která by vedla mimo jiné k lepšímu pochopení ekologického stavu vod.

CONTENT

1. INTRODUCTION

1.1 General diatom characteristics

Diatoms are unicellular autotrophic organisms, belonging into the group of Stramenopiles (Adl et al., 2012). Their typical feature is a silica cell wall – a frustule. They appear all over the world in various aquatic or wet terrestrial habitats. Traditionally they have been divided into two groups according to the valve symmetry: to *centrics* with radial symmetry and *pennates* with bilateral symmetry.

1.2 Diatom age, diversity and ecological functions

Diatoms are a relatively young group of species, existing probably from the early Mesozoic era (Kooistra & Medlin, 1996; Medlin et al., 1997) but they are the most species-rich group of algae (Guiry, 2012; Mann & Vanormelingen, 2013). Today, 14,575 species are described (Guiry & Guiry, 2017), however this number seems to underestimate the overall diversity. There are still undersampled habitats and areas on the earth, such as epipelon (Poulíčková et al. 2008; 2014), subtidal marine epipsammic diatoms (Mann et al., 2016), together with freshwater and aerophytic diatoms in tropical regions. Also, diatoms are substantially underclassified for the cryptic and semicryptic species diversity (Sims et al., 2006). Estimations of the real diversity differ a lot. Mann & Droop (1996) suggest 200,000 existing species, Williams and Reid (2006) claim it is even more than that, Guiry is more conservative with 20,000 species (Guiry, 2012), however, most probably the number lies somewhere between 30,000 and 100,000 (Mann & Vanormelingen, 2013) which still makes diatoms ‚the beetles of algae‘ (Mann & Vanormelingen, 2013). Even though there is such a large number of species, diatoms are still rapidly evolving as they have a very fast substitution rate in rRNA coding regions (Kooistra & Medlin, 1996).

Diatoms, mainly marine planctonic species, one of the main marine planctonic groups (Benoiston et al., 2017), create a huge biomass which gives them great ecological importance. They are oxygen factories and they are responsible for around 20% of the global net primary production (Field et al., 1998; Mann, 1999; Benoiston et al., 2017) and they hugely influence the CO_2 concentration in the atmosphere (Armbrust, 2009). Diatoms participate in the global biogeochemical cycles, mainly of carbon (Benoiston et al., 2017) silica (Tréguer et al., 2013), and nitrogen (Armbrust, 2009).

1.3 Life strategies

Life strategy, the way how a diatom adapts to the environment and to biotic influences, is classified in various ways according to different authors. Mann et al. (2016) divides diatoms into three groups: they live either suspended, attached or they are motile. The latter two, living in the association with the surface, are often called benthic.

1.3.1 Planktonic diatoms

Diatoms living suspended in the water column are called planktonic. They float passively and as they are not actively movable, they might sink thanks to the heavy silica frustule. To stay in more illuminated surface waters, some species create adaptations. They might either form spatially extensive colonies like e.g. *Asterionella formosa* Hassall creating a star, some produce chitin fibres that slow down the sinking (Walsby & Xypoleta, 1977), or incorporate compounds of low density inside their vacuoles like some marine species do (Boyd & Gradman, 2002). However, sinking also represents an important part of the life strategy. When the ocean surface waters turn hostile, diatoms sink and the upwelling brings them back later to better conditions (Smetacek, 1985). The removal of diseased cells can also cause a better accessibility of nutrients (Raven &Waite, 2004). Therefore, diatoms are able to use chitin fibres also for aggregation which accelerates sinking (Smetacek, 1985).

1.3.2 Benthic diatoms

Benthic diatoms are a diverse group of species living close to the substrate. They include motile and non-motile species. Every type of surface is very specific and diatoms are usually divided according to that. Benthic algae are divided into: epipelon, endopelon, epipsammon, endopsammon, epilithon, endolithon, epixylon, epizoon, endozoon, endophyton and finally epiphyton (reviewed by Poulíčková et al., 2008).

Epipelic and *endopelic* diatom communities live in association with fine sediments. They coat the surface of mud, where they can create brownish films. Boundaries between species on and in the sediments do not exist (Poulíčková et al., 2008) as diatoms are usually biraphid and therefore capable of biologically driven circadian or diurnal movements up and down (Palmer & Round, 1967). It is generally thought that the movement up during a day ensures better illumination and down later in the day brings diatoms into contact with higher concentration of nutrients, as nutrients are more accessable in the hypoxic or anoxic conditions. Movement seems to be an essential property in sediment conditions because diatoms are often buried because of disturbances and they can stay active only in the top few millimetres of the sediment (Mann et al., 2016).

Epipelic diatoms are important from the ecological point of view, apart from the primary production they stabilize sediments and participate in the nutrient cycling (Poulíčková et al., 2008).

Diatoms living in the sandy environment are divided into *endopsammon* – living within the substrate like *Surirella* sp. or *epipsammon* – tiny diatoms living attached to a grain of sand, e.g. small representants of *Navicula* sp. and *Nitzschia* sp. (Poulíčková et al., 2008). One grain can be inhabited by up to a hundred diatoms (Mann et al., 2016). As in the case of epipelon, motility represents a great advantage. It is a way of coping with disturbations, and these diatoms are also known to perform vertical movement for the same reasons as epipelon.

Epilithic diatoms live either on the stones, calcified surfaces or similar hard substrata that are regarded as inert. This is a reason why epilithic diatoms are accepted as suitable for biomonitoring purposes. Bacterial activity within the epilithic community, however, causes chemical weathering and therefore makes some elements more available (Hiebert & Bennett, 1992).

The Endolithic way of life is oftenly connected with extreme environments and it is rather typical for other groups of algae, mainly cyanobacteria. However, diatoms are known to live within the rock material. Hernández-Chavarría & Sittenfeld (2006) have even described an endolithic community which was dominated by the diatom *Pinnularia* in volcanic rocks in Costa Rica.

Diatoms are also able to live on or inside animals. *Epizoon* is known from various species, they are found on marine (e.g. Majewska et al., 2015) and freshwater turtles (Wu & Bergey, 2017), sperm whale (Denys, 1997), small crustaceans (Fernandes & Calixto-Feres, 2012) and many others. Algae use animals as vectors for their dispersal (Atkinson, 1972).

Finally, diatoms can live in association with plant material. They can live either inside – *endophytic*, or on the surface – e*piphytic*, creating sometimes very large extensive colonies. Epiphyton, as the key topic of this dissertation, is discussed in detail in the following review, paper I.

1.4 Epiphyton - Paper I

LETÁKOVÁ M., FRÁNKOVÁ M. & POULÍČKOVÁ A. (2018): Ecology and applications of freshwater epiphytic diatoms – review. *Cryptogamie, Algologie* 39(1):1-20.

Ecology and applications of freshwater epiphytic diatoms – review

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Abstract - Epiphytic diatoms perform a variety of ecological functions. Diatoms are important primary producers and sources of oxygen which can modify the chemistry of the surrounding aquatic environment. They may live attached to plant surfaces with the help of extracellular polymeric substances and compete with plants for resources (e.g., light, nutrients). Thus, they represent an excellent model system for studies on interactions between epiphytes and their host plants under different environmental conditions. Further, the practical usage of epiphytic diatoms in biomonitoring begs questions concerning substrate specificity, diatom biodiversity, and species delimitations. This review focuses on specific aspects of freshwater epiphytic diatom ecology as adaptations for epiphytic way of life, epiphyte-host relationships, and implications for biomonitoring.

epiphytic diatoms / ecology / substrate specificity / biomonitoring / species complexes

1. INTRODUCTION

Diatoms live either freely in the water column or attached to substrates. Attached diatoms are differentiated according to substrate type, such as *epipelic* (living on fine bottom sediments; reviewed by Poulíčková *et al.* 2008a; 2014), *epilithic* (growing on stones or hard substrata), *epipsammic* (attached on sandy sediments), *epizoic* (living on animals) and finally *epiphytic* (growing on different plant material such as algae, bryophytes and vascular plants, Round & Lee, 1989; Tiffany & Lange, 2002; Tiffany, 2011).

The need to study epiphytes arises from their ecological importance and functions. Community ratios among primary producers (e.g., macrophytes, phytoplankton, periphyton) are crucial to maintaining a favourable transparent states in lakes with applications in lake management and restoration (Špoljar *et al*., 2017). Attached littoral algal communities represent an important component of food webs as primary producers (Michael *et al.,* 2006). This productivity may be comparable (Wetzel, 1964) or, under certain circumstances, even higher than the productivity of phytoplankton (Vadenboncoeur *et al*., 2003; Adame *et al*., 2017). We speak mainly about oligo-mesotrophic shallow lakes or littoral zones (Vadenboncoeur *et al.*, 2003; 2008; Althouse *et al.*, 2014) where the transparency of water is higher and enables periphyton development. This may also be the case in lotic systems (mainly in mid-sized streams), where the primary production of epiphyton is considerable (Vannote *et al.*, 1980).

Attached algae, similar to submerged macrophytes, chemically modulate the aquatic environment by nutrient uptake and assimilation-dissimilation processes (Lock *et al.* 1984; Celewicz-Gołdyn & Kuczyńska-Kippen, 2017) Epiphytic diatoms actively metabolize and therefore change chemical compositions and ratios in water, while their photosynthetic activity leads to diurnal fluctuations of oxygen and CO_2, with subsequent pH variation (Lelková & Poulíčková, 2004). Although the ability of atmospheric nitrogen fixation is mainly connected with cyanobacteria, some diatoms can participate in nitrogen cycling via their endosymbionts (i.e., members of the family Rhopalodiaceae, *sensu* Precht *et al*., 2004; Nakayama *et al.,* 2014). Finally, epiphytic diatoms play an important role in bioindication and paleoecological reconstructions (Kitner & Poulíčková, 2003; Poulíčková *et al.*, 2004a; Denys, 2009; Poulíčková *et al*., 2013b).

Although the attached photoautotrophs have received less attention than the phytoplankton, the number of epiphyton studies exceeds studies of other bentic habitats. The ratio of papers in Web of Knowledge database (Thomson Reuters, New York) on planktonic, epiphytic, and epipelic microalgal assemblages was 62:32:4 in 2013 (Poulíčková *et al.*, 2014). However, there exist knowledge gaps which restricts the practical applications based on epiphytic assemblages. The purpose of this review is to address some specific aspects of freshwater, epiphytic diatom ecology, plant-diatom relationships within epiphytic communities, implications for biomonitoring, and to suggest the directions for future research.

2. LIFE ON THE SUBSTRATE

An attached growth form provides periphytic diatoms several advantages over phytoplankton – stability, light and nutrient access. In an ecological niche, phototrophic organisms compete for light and only those with suitable adaptations can be successful. Light appears to be the overriding factor controlling both macrophyte and epiphyton biomass, composition, and distribution (Mosisch *et al.* 2001; Laugaste & Reunanen, 2005; Pettit *et al.,* 2016). The relative cover of periphyton decreases with decreasing light, which means that light conditions influences the vertical distribution of photoautotrophs (Poulíčková *et al.,* 2006). Periphytic diatom species are low light tolerant, which could explain their higher abundance at sites with high overshading (Celewicz-Gołdyn & Kuczyńska-Kippen, 2017).

Attachment enables epiphytes to stay in places with optimal light conditions and perhaps in the vicinity of decaying plant cells, which may serve as a nutrient source (Cattaneo & Kalff, 1979). However, competition for such surfaces is stiff even amongst the periphyton itself (Hoagland *et al.*, 1982), favouring motile diatoms (Hudon & Legendre, 1987). Secondly, attachment to the surface prevents current stress, mainly to diatom cells living closest to the substrate (Stevenson, 1996). Passy (2007) classified attached diatoms into three guilds: low-profile, high-profile, and motile diatoms (Fig. 1-15). Low profile representatives have small body size, horizontal growth, and tolerate unfavourable positions close to the substrate (Fig.4-5). Both the high-profile and motile guilds may secure more beneficial positions due to specialized habits (Fig.6-7, Soininen *et al.*, 2016). As planktonic diatoms can settle into the benthos, a fourth “planktic” guild has been added (Rimet & Bouchez, 2012b) to the classification mentioned above (Fig.16).

Fránková *et al*. (2017) divided epiphytic diatoms in a similar way. They created five functional groups according to their relation to the host plant surface (“life style“): FT1 planktonic taxa represented by centric diatoms; FT2 typically periphytic taxa adhering to the surface directly by a mucous film or with a mucilaginous stalk; FT3 facultatively periphytic araphid taxa, passively moving diatoms able to attach; FT4 facultatively periphytic taxa with raphe with fibulae, actively moving diatoms able to attach; FT5 epipelic taxa with raphe, actively moving, mainly symmetrical biraphid pennate diatoms.

2.1 Attachment to living surface

Diatoms should not be considered parasitic since they stick to the external tissue only, with the help of extracellular polymeric substances (EPS, Tiffany, 2011; Fránková *et al.,* submitted), that are considered to be one of the reasons for diatom ecological success. Because, apart from sessile adhesion, they enable a variety of other functions such as motility), colony formation and they also serve as antidessicants (Hoagland *et al.*, 1993). EPS are composed mainly of acidic polysaccharides (Wustman *et al.*, 1998), more or less carboxylated or sulphated, while more detailed composition is genus- or species specific (Hoagland *et al.*, 1993). Occasionally, proteins have been identified as well (Daniel *et al.*, 1987; Wustman *et al.*, 1998), but never lipids (Hoagland *et al.*, 1993).

EPS may form a variety of structures, such as stalks, pads, and adhering films, all of which are crucial for attachment (Fig. 1-16). *Stalks* are more or less long fine fibrils connecting the substrate and diatoms. They are exuded from the siliceous cell wall either through the apical pore field (Hoagland *et al.*, 1993, e.g., *Cymbella cistula* (Ehr.) Kirchn. (Hufford & Collins, 1972), *Cymbella affinis* Kützing (Roemer *et al.*, 1984), *Gomphonema olivaceum* (Hornemann) Brébisson (Roemer *et al.*, 1984), *Rhoicosphaenia curvata* (Kütz.) Grun. (Mann, 1982), etc.), or from the raphe, at the terminal nodule on the valve face (Hoagland *et al.*, 1993, e.g. *Achnanthes longiceps* (Daniel *et al.*, 1987; Novarino, 1992) and *A. minutissima* Kütz (Roemer *et al,* 1984). *Pads* do not differ much from stalks: they are even called ‚short stalks‘. They are also secreted through the apical pore field (Hoagland *et al.*, 1993). However, apart from the attachment to the surface they enable also the cell to cell attachment and therefore are responsible for colony formation (Geitler, 1971). Diatoms forming pads include *Fragilaria acus* Kütz. and *Gomphonema parvulum* (Kütz.) Grun. (Hoagland *et al.*, 1982), *Fragilaria vaucheriae* (Ehr.) Peter (Roemer *et al.*, 1984), *Diademis confervacea* Kützing (Rosowski *et al.*, 1983), etc. Adhering films are found in prostrate diatoms such as *Amphora* (Daniel *et al.*, 1980, Round & Lee, 1989), and *Cocconeis* (Daniel *et al.*, 1987, Wang *et al.*, 2014).

The movement of chemotactic diatoms is influenced by the chemical nature of the substrate (Chet & Mitchell, 1976). This chemotaxis can be passive or active, as observed on the marine diatom *Achnanthes longiceps* (Wang *et al.*, 1997). Diatoms inoculated to the hydrophobic substrate attached passively and reversibly but diatoms inoculated to hydrophilic substrate did not attach until they produced extracellular polymers. Active attachment can be observed on the hydrophobic surface as well. However, much more time is needed and the action has four steps. Raphe-associated transient attachment enabling movement is followed by the formation of pads which prevents motility. Then, a shaft is secreted from the pole of the raphe valve and it elongates. Finally, the cells at the end expand creating colonies (Wang *et al.*, 1997).

Like higher plant assemblages, attached algal succession appears to be a result of interactions among processes such as colonization, environmental filtering, interspecific, and intraspecific competition (Hoagland *et al.*, 1982; Kitner *et al.*, 2005; Passy & Larson, 2011). As Hoagland *et al.* (1982) describe, firstly the substrate becomes coated with organic film and the bacteria attach, sometimes even actively via mucilaginous material. The presence of bacteria definitely plays an important role, as Buhmann *et al*. (2011) note that *Achnanthidium minutissimum* produces mucilaginous material only if bacteria are present. Second, early colonizers may be various types of microbes, including low-profile diatoms. Later, clumps or rosettes of diatoms develop, finally followed by long stalked (high profile) diatoms. Their stalks represent a surface for further epiphytic attachment (secondary epiphytes) and therefore further structuring (Fig. 11-12). Secondary epiphyton species richness, abundance and even biomass (Whitton *et al*., 2009) can exceed quantitative parameters of primary epiphyton (Letáková, unpublished data). The arrangement of epiphytic assemblages in the climax stadium of succession resembles the multi-layered structure of forests (Kitner *et al.*, 2005; Poulíčková *et al.*, 2006).

The microbial community has a diffusive boundary around itself (Jørgensen & Revsbech, 1985), where the chemical conditions inside may differ significantly from the surrounding environment and affect the metabolism of the whole community (Carlton & Wetzel, 1987; Riber & Wetzel, 1987). The older the community, the bigger the difference in chemical conditions where the exchange of chemicals from an older community with external water is significantly less important than that of a young and thin community (Sand-Jensen, 1983).

2.2 Host plant surface

Diatom flora is influenced by the physical, chemical, and biological characteristics of the host plant surface, even though there is not a strict boundary between these influences. Biological interaction is often run by chemical substances and the physical one is often associated with the chemical one, etc. The section is divided according to these three interconnected aspects.

2.2.1 Physical influence

The physical influence of the host surface is represented mainly by macrophyte host morphology (overall body architecture, form of leaves, etc.), anatomy (particularly surface microtopography, e.g. roughness or smoothness), and growth forms (emerged, submerged and floating). The length of host life cycle is also important (Laugaste & Reunanen, 2005; Pomazkina *et al.*, 2012; Letáková *et al.*, 2016; Pettit *et al.,* 2016).

Primarily, diatom composition is influenced by the size and surface texture (Whitton, 1975), which is most evident in lotic waters where it is harder to stay attached, but it is present also in lentic waters (Laugaste & Reunanen, 2005), highlighted in splash zones. Finely branched and morphologically complex submerged plants, such as *Myriophyllum, Ranunculus* or *Elodea,* seem to be rich in epiphytes (Laugaste & Reunanen, 2005; Celewicz-Gołdyn & Kuczyńska-Kippen 2017). Similarly, higher diatom density was found on bryophytes with more crevices than on leafy liverworts (Knapp & Lowe, 2009) because the current is decelerated within the bryophyte thallus (Suren *et al.*, 2000) creating a shielded habitat enabling higher attachment rates (Burkholder, 1996). Similarly, in the study conducted on the River Durance in South-East of France, significant differences in epiphyton densities were observed in different parts of plants (Compte & Cazaubon, 2002). The influence of water movement is also known from lentic ecosystems. Albay & Akçaalan (2008) show that physical disturbances, such as water-level fluctuation, influence colonisation of epiphyton. Fránková *et al*. (2017) found in the Dehtář fishpond (the Czech Republic) different compositions of epiphytes according to their functional traits (diatom “life style“ in relation to the substrate) caused by different intensity of wave action.

Epiphytic species composition may also differ significantly between various macrophytes taken under similar conditions (Compte & Cazaubon, 2002; Fernandes *et al.,* 2016; Mutinová *et al*., 2016). Diatoms have diverse body shapes, sizes, and means of attachment, and for thus heterogeneity of colonization may be most prominent in the presence of a current, but again it appears also in lentic waters. For instance, large adnate diatoms, such as *Epithemia adnata, Rhopalodia gibba, Eunotia arcus, E. arcubus,* are the most frequent and abundant inhabiting *Potamogeton gramineus* rather than *Chara aspera*, which are favored mainly by small-sized and motile taxa – such as *Brachysira neoexilis* and *Encyonopsis cesatii* (Laugaste & Reunanen, 2005; Letáková *et al.*, 2016). Micro-scale distribution pattern of periphyton taxa is associated with microhabitats and influences the overall distribution and diversity of benthic autotrophs (Yang *et al*., 2009). Such fine-scale distributions can finally be studied using new and promising tools for *in vivo* observation (Fig. 1-3, Fránková *et al.*, 2017). For example, Low Temperature Method for Environmental Scanning Electron Microscopy (LTMESEM) enables the study of fresh diatom material attached on plant substrate without any chemical pretreatment and conductive coating.

The variation of diatom epiphytic assemblages can be expected in consequence of host plant life forms as emergent macrophytes (e.g., *Typha spp.*), submerged macrophytes (e.g., *Myriophyllum*), free floating macrophytes (e.g., *Lemna*), macrophytes with floating leaves (e.g., Nymphaceae), and wet bryophytes (Poulíčková *et al.,* 2004b; Fernandes *et al*., 2016; Letáková *et al*., 2016; Adame *et al.,* 2017). Floating-leaved macrophytes increase shading, reduce both planktic and bentic algal/diatom photosynthesis and reject zooplankters, whilst emergent macrophytes prevent coastal erosion (reviewed by Špoljar *et al*., 2017). In summary, a mosaic structure of macrophyte community caused higher habitat heterogeneity and support overall diversity (Wang *et al*., 2009). Over the last two decades, increasing attention has been paid to bryophytes because of the ecological significance of wetlands. Mosses often have unique diatom flora, therefore Johansen (1999) uses the term *bryophytic diatoms* and Cantonati named epiphytic algal assemblages inhabiting mosses *epibryon* (Cantonati *et al.*, 2012; Poulíčková *et al*., 2013a). The moss thallus protects the epiphyton from heat, wind, and desiccation since it is able to retain a great amount of water. Moisture content is the major ecological parameter determining diatom assemblages in moss communities, especially in the terrestrial environment (Nováková & Poulíčková, 2004; Poulíčková *et al*., 2004b; 2013a). Epibryon (diatoms on bryophytes) is common and abundant in regions where mosses comprise the dominant vegetation: Subarctic and Subantarctic islands (Van de Vijver & Beyens, 1997; Van de Vijver *et al.*, 2003; Chattová *et al*., 2014), peat bogs and mires (Poulíčková *et al*., 2004b; Buczkó & Wojtal, 2005; Buczkó, 2006; Kokfelt *et al*., 2009; Poulíčková *et al*., 2013a,b) and spring fens of boreal and temperate zones (Cantonati 1998; Poulíčková *et al*., 2003; 2004b; Fránková *et al.*, 2009; Hájek *et al*., 2011; Cantonati *et al*., 2012).

2.2.2 Chemical influence

Various chemical substances influence benthic diatoms with two main groups bearing note: nutrients and allelopathic substances.

Apart from nutrients supplied via the water column, benthic diatoms may also obtain nutrients from underlying substrata (Burkholder & Wetzel, 1990). The use of nutrient-diffusing artificial substrates has shown that benthic diatoms receive nutrients from underlying substrata, (Fairchild *et al.*, 1985; Carrick & Lowe, 1989; Pringle, 1990), particularly phosphorus (Burkholder & Wetzel, 1990), silica (Sand-Jensen, 1990) and organic matter (Kassim & Al-Saadi, 1995). The prevailing source also influences the community structure and species diversity (Pringle, 1990). Substrates releasing nutrients were described in the epipelon (Pringle, 1990; Hašler *et al.*, 2008; Poulíčková *et al.*, 2008a; 2014), in studies on artificial substrates (Fairchild *et al.*, 1985; Carrick & Lowe, 1989), and also in freshwater epiphyton (Burkholder & Wetzel, 1990) particularly in the early stages of colonization (Albay & Akçaalan, 2003). Host-plant nutrient supplies play an even more important role in habitats with low turbulence of water because water flow enables nutrient cycling that diffuse across a thin boundary level (Riber & Wetzel, 1987).

In contrast to the oldest studies suggesting macrophytes as an inert material (Cattaneo & Kalff, 1979), Burkholder & Wetzel (1990) consider host plant surface as important nutrient source for epiphyton. The importance of this nutrient source increases particularly in oligotrophic and moderately eutrophic waters (Kitner & Poulíčková, 2003; Poulíčková *et al*., 2004a; Laugaste & Reunanen, 2005).

The release of nutrients from plants is pronounced on sites of tissue damage, where more epiphytes may grow (Roos, 1983). Also, with increasing plant age, the integrity of the material decreases and therefore more nutrients are diffused, particularly phosphorus (Landers, 1982) or silica (Laugaste & Reunanen, 2005). Furthermore, the release of nutrients is greater in the spring time after winter decomposition than in the late summer time. However, no matter the age and seasonality, there is always nutrient release to some extent (Burkholder & Wetzel, 1990). Unfortunately, new studies in this field are missing, although new methods and sophisticated facilities have recently become available.

2.2.3 Biological interactions

Life on a plant substrate is usually regarded as beneficial for diatoms: their gain is quite clear. As it is mentioned above, they acquire a better position in the water column, higher light illumination together with a wider source of nutrients. But the question is: How do epiphytes influence their hosts? Different studies often support completely opposite opinions.

2.2.3.1 Negative influence of hosts

At first, the epiphyte vs host macrophyte relationship can be regarded as negative. Epiphytes shade aquatic plants and therefore decrease their photosynthetic activity (Philips *et al.*, 1978; Eminson & Moss, 1980; Sand-Jensen & Borum, 1984). Epiphytes also compete with macrophytes for space and nutrients, they increase pH and oxygen levels, and during the night, they contribute to the induction of hypoxic conditions (Sand-Jensen & Borum, 1984). Consequently, aquatic macrophytes have developed certain protective adaptations: either a smooth surface (Eminson & Moss, 1980), quick growth of new tissue/leaves and changes in macrophyte morphology (Eminson & Moss, 1980; Sultana *et al*., 2010), or the production of algal antibiotics (Mähnert *et al*., 2017). Changes in leaf shape were reported in response to water depth and phosphorus limitation (reviewed by Sultana *et al*., 2010). The average number of leaves, total length of newly recruited shoots, and diameter of stems seems to be greater in the epiphyton-free control plants than in the epiphyton-colonized plants under low light conditions (Sultana *et al*., 2010). They also showed that in laboratory conditions plants with epiphyton allocate more biomass in their rhizomes and roots. In contrast to epiphyton free plants, epiphyton-laden plants did not show internodal elongation, which is considered as a response of plant to red light under shading conditions (Sultana *et al.,* 2010). It is known that primary producers can produce allelopathic substances in order to beat rivals (Gross, 2003; Mähnert *et al*., 2017). The toxic influence on cyanobacteria and algae has been shown experimentally in case of *Myriophyllum* (Gross *et al.,* 2002), *Ceratophyllum* (Iványi *et al.,* 2002), and *Chara* (Mähnert *et al*., 2017). Allelopathic effects of submerged macrophytes on phytoplankton have been studied for their practical usage in water management (Körner & Nicklisch, 2002; Gross *et al.*, 2007; Hilt & Gross, 2008; Hu & Hong, 2008). Epiphyton is considered to be less vulnerable to allelopathic chemicals than phytoplankton (Hilt, 2006), probably because epiphytic algae might have developed resistance to some extent by the process of co-evolution (Reigosa *et al*., 1999).

Allelopathic substances are not only those with an inhibitory effects but also stimulatory ones (Molish, 1937), although the latter are the main discussed in the literature (Letáková *et al.*, 2016). Understanding allelopathic substances still represents a challenge because elucidating their roles and prevalence in an aquatic environment is not an easy task, and the laboratory evidence does not necessarily apply in nature (Gross, 2003; Berger & Schagerl, 2003; 2004; Mähnert *et al*., 2017). The question is also if the chemicals are secreted in biologically active amounts and if they are secreted ‘intentionally‘ or just as a coincidence of factors, to know if they should be called toxins or allelopathic substances (Inderfit & Dakshini, 1994). These are the reasons why this chemical-biological issue still requires further investigations.

A typical group studied for allelopathic release are Charophytes. They produce toxic substances inhibiting the photosynthesis of microalgae, which together with the incrustation of their surface by calcium carbonate is responsible for the lower epiphyton densities on *Chara* spp. (Hafner & Jasprica, 2013; Letáková *et al.,* 2016). Allelopathic substances produced by Charophytes are known to lower the photosynthetic rates (Dodds, 1991) or even completely inhibit photosynthesis of different species of the genus *Nitzschia* (Wium-Andersen *et al.,* 1982). Fránková *et al.* (2017) observed lower diatom species richness on the non-incrusted *Chara braunii* (20−24 diatom species per sample) in comparison with *Elatine hydropiper* (44−46 diatom species per sample) studied at the same locality. The species of the genus *Nitzschia* present on other macrophytes were almost absent. On the other hand, stoneworth epiphyton from south Bohemian fishponds was inhabited by common euryvalent species, which probably have a high tolerance to stress factors (Fránková *et al.,* 2017).

Low epiphyte density is also known from *Spirogyra*. Tannin-like compounds released by this filamentous macroalga might be responsible for this (Pankow, 1961). Jorgensen (1956) notes that *Chlorella pyrenoidosa* filtrate inhibits the growth of *Nitzschia palea*, while the same substance stimulates the growth of *Desmodesmus quadricauda*. *Nitzschia palea* produces an autotoxic substance, while *Asterionella formosa* forms a substance accelerating its own growth (Jorgensen, 1956). Unfortunately, the evidence of influences of chemicals secreted by macrophytes or other benthic organisms on diatoms and chemicals secreted by diatoms themselves is greatly lacking.

2.2.3.2 Neutral influence

Not all consider the macrophyte – diatom relationship to be determinative for the occurrence of diatoms species. According to the ‘neutral substrate hypothesis,‘ there is no significant effect of the plant substrate on its epiphytes (Cattaneo & Kalff, 1979; Blindow, 1987; Kitner & Poulíčková, 2003; Cejudo-Figueiras *et al.*, 2010) and algal communities growing on surfaces other than plants do not differ greatly. Neither plant nor diatom influences are essential, and, although macrophytes provide some nutrients to epiphytes, their influence can be neglected (Cattaneo & Kalff, 1979), especially in waters with high trophy. If a water plant is substituted with an artificial substrate, the diatom community does not differ significantly (Cattaneo & Kalff, 1979; Millie & Lowe, 1983).

2.2.3.3 Positive/symbiotic interactions

Finally, there are studies suggesting that this type of relationships can be regarded as mutualistic (Ulanowicz, 1995). Epiphytes have been defined as loose non-obligate ectosymbiotic (Allen, 1971; Wetzel, 1975; 1983; Kies, 1992) though it sounds too bold after all the facts mentioned above. We already know what the diatom gains, but what advantage can epiphytic diatoms bring to their hosts? If the shading effect of epiphyton is not too heavy, it brings the benefit of absorption of UV radiation and therefore protects the macrophytes (Klančnik, 2014). There is one more important factor within the water environment and that is grazing pressure. Aquatic plants and macroalgae are under the risk of consumption. Epiphytic cover can represent a barrier against host grazers (Hutchinson, 1975), since they are eaten first and the aquatic plant might stay unhurt. Grazing pressure strongly influences the epiphytic community and it prevents the undesirable shading (Brönmark, 1989; Hillebrand, 2005).

3. EPIPHYTIC DIATOMS IN BIOMONITORING

3.1. Overall diversity

Biomonitoring is based on specific ecological requirements of organisms called bioindicators (Adams, 2002). Aquatic bioassessment based on phytobenthos, particularly diatoms, seems to be quite popular especially in freshwater ecosystems (Lavoie *et al*., 2014). However, major taxonomic revisions clearly showed that the overall diversity of diatoms is underestimated. Although we have 12,000 currently described diatom species, the estimates of total diversity range between 30,000 and 200,000 (Mann & Vanormelingen, 2013). Recent evidence from molecular data and mating experiments has shown that some traditional morphospecies are aggregates and contain several cryptic species (e.g., *Achnanthidium minutissimum* agg.), which are ecologically differentiated (Poulíčková *et al*., 2008c; 2017). Increasing numbers of described diatom species lead to problems with their usage in routine biomonitoring (Zampella *et al.,* 2007). Analyses based on epiphytic assemblages lead to the recommendation employ fine taxonomic resolution in cases where the assemblage is dominated by a “good indicator”, which is an easily recognized diatom taxon (Poulíčková *et al*., 2017). Species as *Achnanthidium minutissimum* (Kützing) Czernecki, *A. lineare* W. Smith and *A. caledonicum* (Lange-Bertalot) Lange-Bertalot seems to be promising as indicators of lower trophic levels and *A. eutrophilum* (Lange-Bertalot) Lange-Bertalot and *A. straubianum* (Lange-Bertalot) Lange-Bertalot as indicators of higher trophic levels.

3.2 Host specificity

The issue of substrate preference arises from physical, chemical, and biological influences. Since the relationship between epiphyte and macrophyte is complex, the idea of substrate specificity is intriguing. The fundamental question is if the environmental conditions or the host type are more responsible for the community structure. The answer has obvious relevance for the use of diatoms in biomonitoring. Conflicting data has been generated from different studies, localities, and macrophyte species.

Prowse (1959) suggests there is a probable relationship between macrophytes and their epiphytes, i.e. certain species of macrophytes have certain species of epiphytes. This might be due to different surface architecture (Lauguste & Reunanen, 2005), possible allelopathic release (Gross, 2003; Hilt, 2006), or for other biotic interactions (e.g., Pip & Robinson, 1984; Cattaneo *et al.*, 1998; Laugaste & Reunanen, 2005; Letáková *et al.*, 2016). Some researchers have noted host specificity only for certain macrophytes while not for others (Blindow, 1987; Kollár *et al.*, 2015; Mutinová *et al.*, 2016; Messyasz & Kuczynska-Kippen, 2006). In a similar way, the diatom *Lemnicola hungarica* is recorded as a typical inhabitant of *Lemna* sp. (Buczkó, 2007; Poulíčková *et al*., 2008b). However, *Lemnicola* has also been recorded from *Phragmites australis* (Kollár *et al.*, 2015). *Cocconeis placentula* is well known from algal surfaces, particularly *Cladophora-Cocconeis* association (Laugaste & Reunanen, 2005; Tiffany 2011, our observations Fig.4-5). On the other hand, negligible or no substrate-dependent differences have been noted (e.g., Cattaneo & Kalff, 1979; Milie & Lowe, 1983; Cejudo-Figueras, 2010).

Related to this issue, most studies agree that host specificity is usually observed in places with lower trophy (e.g. Eminson & Moss, 1980; Poulíčková *et al.*, 2004a; Letáková *et al.*, 2016; Mutinová *et al.*, 2016). The higher the trophic status the less the specificity, which, if present, is likely due to plant morphology (Eminson & Moss, 1980; Kitner & Poulíčková, 2003). For example, Messyasz & Kuczyńska-Kippen (2006) documented the substrate preference in eutrophic lakes and concluded that the specific architecture of a host plant is more determinative than the features of the lake. In oligotrophic waters, the possible release of nutrients by plant surface might play a more important role than in waters full of inorganic and organic compounds (Eminson & Moss, 1980; Kitner & Poulíčková, 2003). However, some studies claim the opposite (Kahlert & Peterson, 2002).

In the case of bryophytes, water chemistry is still found to be the most relevant determinant. For example, *Sphagnum* seems to influence epiphyton by altering the pH (acidification) in the surrounding environment (Clymo, 1964; Mutinová *et al.,* 2016), although the effect is not universal. Although some authors found a significant relationship or affinity between some diatom species and bryophytes (Cantonati, 1998; Poulíčková *et al.*, 2004b; Cantonati & Spitale, 2009), the environment (especially water chemistry represented mainly by pH) appears to be more important than the substrate (Buczkó, 2006; Cantonati & Spitale, 2009; Hájková *et al*., 2011).

In sum, host preference is an unresolved issue relating to epiphytic diatoms. The chemical and biological interaction between the host plant of various species and its epiphyton should be in the centre of attention, although it is problematic from the practical point of view.

3.3 Biomonitoring and limnological reconstructions

Water is an essential substance for humans and therefore there is a need to maintain and protect it. These actions are impossible without deep understanding of what constitutes a “healthy” and “natural state”. To analyse such complex systems, chemical analyses need to be accompanied by the biological ones (Round, 1991): the chemical analysis illuminate the immediate information about the environment, while organisms provide complex and long-term information, as they need some time to develop their community. Furthermore, simple chemical analysis may be biased by the organisms that cause the fluctuations, thus the simultaneous usage of bioindicators is necessary (Cox, 1991; Round, 1991). Among other chemical and biocenotic investigations, diatoms seem to show the most precise results (Leclercq, 1988; Hájek *et al.,* 2014; Rimet *et al*., 2015; Kelly *et al*., 2016). The composition of the community reflects various physico-chemical characteristics (van Dam, 1982), since diatoms have preferences for pH, conductivity, humidity, trophy, presence of organic matter, oxygen, nutrients, current and so on (Lobo *et al.*, 2016). Diatoms are perfect bioindicators (Blanco *et al.*, 2004; 2014) that help to distinguish eutrophication (Descy & Coste, 1990; Kitner & Poulíčková, 2003; Poulíčková *et al.*, 2004a) and pollution (Lange-Bertalot, 1979). Moreover, their response to changing conditions is fast (Blanco *et al.*, 2004), even faster than that of macroorganisms (Rühland *et al.,* 2003; Hájek *et al.*, 2014). Therefore, diatoms are among the organisms mentioned in the Water Framework Directive (WFD; 2000/60/EC) as fundamental bioindicators of waters.

Periphyton based monitoring must be segregated by ecosystems (e.g., lotic, lentic waters, and wetlands), because different factors apply in each. Many river periphyton studies exist (e.g. Whitton & Rott, 1996; Prygiel *et al.*, 1999; Rimet & Bouchez, 2012a, b) and there is also a standardized sampling method available (CEN, 2003). For ponds and lakes the number of studies is increasing lower (Kelly *et al.*, 2008; 2016), and even more scant for wetlands (Della Bella *et al.*, 2007; Chen *et al.,* 2016). Overall, the epiphytic community has a great potential for biomonitorning of all the three habitat types.

In running waters, the most commonly sampled substrates are epilithon and epiphyton (CEN, 2003; King *et al.*, 2006), with epilithon preferred if present (Round, 1991; Álvarez-Blanco *et al.*, 2013), although in slower parts of a stream it can be contaminated with mud (Round, 1991). The use of epiphyton for biomonitoring purposes also has its difficulties. There are numerous species of macrophytes growing along streams, and a comparison between epiphytes growing on different macrophyte species might be burdened by an error, for the reasons mentioned above. The comparison of epilithon and epiphyton has been done several times for both streams and lakes (Danilov & Ekelund, 2000; Poulíčková *et al.*, 2004a; Torrisi *et al.,* 2006).

In lentic waters, mainly in shallow lakes and littoral parts of deep lakes (Kitner & Poulíčková, 2003; Blanco *et al.*, 2004; Poulíčková *et al.*, 2004a; Ács *et al.*, 2005; King *et al.*, 2006; Blanco *et al.*, 2014; Cejudo-Figueiras *et al.,* 2010), epiphyton was found to be suitable for bioindication, and actually very similar principles can be applied as in lotic systems (King *et al.*, 2006). Cantonati *et al*. (2012) proposed epibryon for the assessment of spring habitats. Epilithon is tied to substrate preferences, but contamination by dead frustules may lead to biased results (Poulíčková *et al.,* 2004a). This can be prevented by using epiphytic samples from vertically orientated macrophytes (reeds), where covering by silt is minimized (see Round, 1991; Poulíčková *et al.*, 2004a; King *et al.*, 2006).

Epiphytic diatoms can be further used in paleolimnological reconstructions. The Water Framework Directive (WFD; 2000/60/EC) requires researchers to derive ecological status categories from „reference conditions“. The sheer absence of reliable reference sites all over Europe, and difficulties with sediment-based paleolimnology limited by poor stratigraphic resolution due to sediment mixing, has inspired some authors to look for other sources of information on a pristine situation (Denys, 2009). Macrophytes that are stored in a herbarium together with their diatom epiphytes represent an easy way to reconstruct past conditions from the composition of their epiphytic assemblage (van Dam & Mertens, 1993; Cocquyt & De Wever, 2002; Shirey *et al.*, 2008; Denys, 2009; Poulíčková *et al.*, 2013b).

4. CONCLUSIONS

We have outlined a number of knowledge gaps with respect to the ecology and importance of freshwater epiphytic diatoms as bioindicators. Future research directions are summarized below:

1. Studies on epiphytic species diversity, distribution, dispersal, and autecology, together with improvements in taxonomy using a combination of molecular and traditional methods with emphasis to compile taxonomic reference libraries for environmental barcoding, should ameliorate limits of biomonitoring systems.
2. Studies on microhabitats and fine-scale distribution of epiphytic taxa "*in situ*" should bring better understanding of diatom life strategies and adaptations.
3. Studies on chemical influences and exchanges within host-epiphyte system, as well as biological interactions (e.g. allelopathy), is necessary for better understanding of host-epiphyte specificity.

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Fig. **1-3** Epiphyton on plant epidermis imaged using environmental scanning electron microscopy (ESEM; method described in Fránková *et al*., submitted): **1**. *Cocconeis* (asterisk), *Gomphonema* (arrow), **2**. *Cocconeis*, *Planothidium*, **3**. *Gomphonema* attached by mucilagenous stalks (arrow).

4-12, 15-16 Epiphytic diatoms imaged using light microscopy (bright field **4-5,11-12,15-16** or Nomarski contrast **6-10, 13-14)** **4,5** – *Cocconeis* (arrow) attached to *Pleurosira* and green alga respectively by valve face (low profile guild), **6-7** *Gomphonema* attached by mucilagenous stalks (arrow, high profile guild), **8-9** *Achnanthidium* attached by mucilagenous stalk, **10**. *Fragilaria* attached to green alga by one end using mucilage pad (arrow), **11-12** *Didymosphenia* with *Achnanthidium* as secondary epiphytes on their stalks (photo Sarah Spaulding), **13**. free living *Navicula* (motile guild), **14**. *Tabellaria* colonies connected by mucilage (arrow), **15**. *Fragilaria* and **16**. *Asterionella* planktonic guild.

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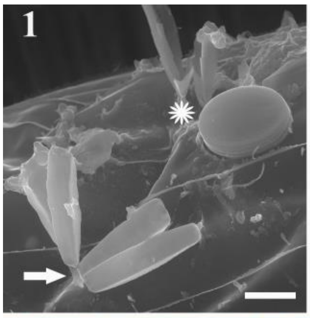

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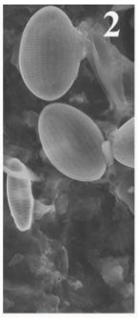

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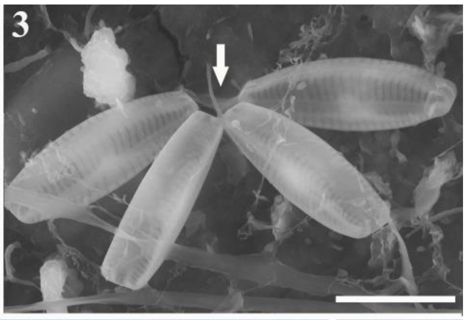

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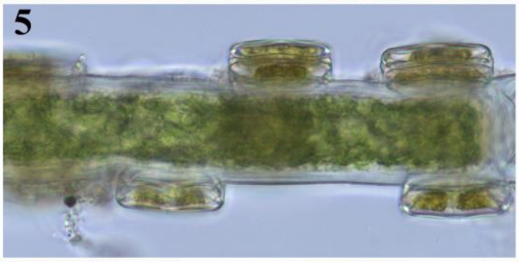

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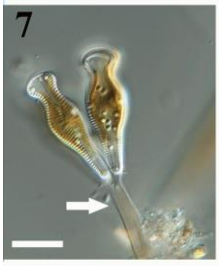

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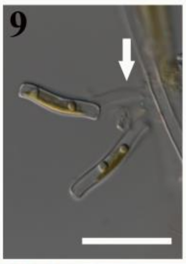

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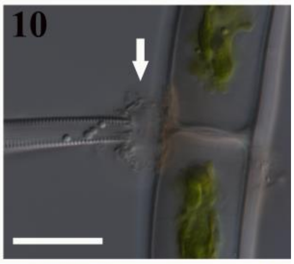

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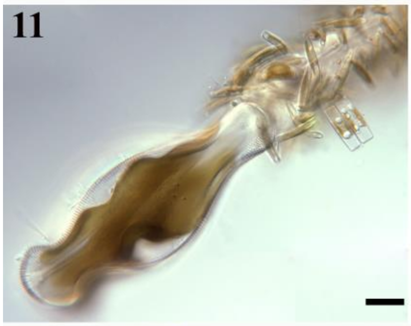

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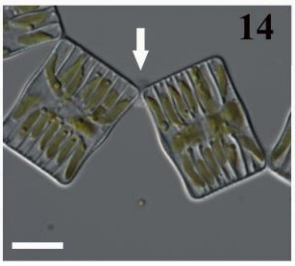

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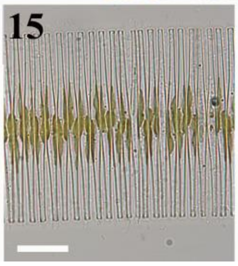

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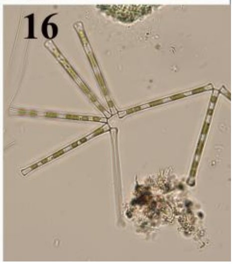

2. OBJECTIVES OF THE DISSERTATION

This dissertation focuses on the community of epiphytic diatoms in order to account for the knowledge about life on the plant substrate and it tries to connect this with the practical aspects important in biomonitoring. In the centre of interest are the following aims:

1) To investigate structure, diversity and composition of epiphytic diatom communities.
2) To study the influence of ecological parameters to the epiphytic diatoms composition.
3) To try to summarize and clarify the problematics of the substrate specificity and its consequences for biomonitoring.
4) To investigate the contribution of species complexes and cryptic/semicryptic diatom species to overall epiphyton diversity.
5) To compare how species complexes and other species follow the trophic gradient and to find out how the accuracy of diatom identification influences water monitoring.
6) To point out epiphytic species complexes whose resolving has the potential to meliorate biomonitoring.

3. METHODS

3.1 Material, sampling and preparation

Epiphytic diatoms with their substrate (microphytes and macrophytes) were collected from altogether 79 localities. They were mostly represented by shallow fishponds and small local streams of the Czech Republic (Kollár et al., 2015; Poulíčková et al., 2017). For the Letáková et al. (2016) study, samples were taken in Lake Valagola in the Brenta Dolomites in Italy. Basic characteristics of each place are given in the individual papers.

Sampling always started when the submersed part of the plant was cut with the scissors and placed into a plastic container. In the laboratory, samples were worked out within few hours. Macrophytes were cut into smaller pieces, they were placed in the Erlenmeyer flasks and filled with around 300 ml of 30% H_2O_2 (volume differed according to the amount of plant material) in order to start the slow oxidation. All was done with great attention to prevent contaminations. After few days, the samples were boiled until the volume decreased considerably and a small amount of $K_2Cr_2O_7$ and 1 ml of conc. HCl were added. In such a way the oxidation process was finished. The diatom samples were washed out in distilled water several times until they reached neutral pH. The appropriate concentration of clean diatom frustules was prepared, and permanent samples were mounted with Naphrax.

Diatoms were observed using a light microscop Zeiss ‘Primo star‘(Germany) and Zeiss Axioskop 2 (Zeiss, Jena, Germany) with phase contrast and images were taken using an Axiocam digital camera. All the samples were investigated qualitatively and semi-quantitatively. For the later one, 400 diatom valves were counted within each sample. Diatoms were identified using the following diatom determination keys: Krammer, (2000, 2002, 2003), Lange-Bertalot (2001), Levkov (2009), Lange-Bertalot et al. (2011), Hofmann et al. (2013), Lange-Bertalot et al. (2017). Nomenclature has been unified according to AlgaeBase (Guiry & Guiry, 2015a; 2015b; 2016).

For SEM observation, clean diatom frustules or herbarized materials were mounted on aluminium stubs, coated with gold and observed in Scanning Electron Microscope Zeiss EVO 40 XVP Zeiss.

Fresh algal assemblages were observed on a stem epidermis using the LTM for ESEM without any pretreatment. Samples were observed using the FEI ESEM QUANTA 650FEG with beam energy 20 kV, probe current 35 pA and working distance 8.5 mm. The method is described in more details in Paper II.

3.2 Measuring ecological parameters

Ecological parameters including temperature, pH and conductivity were measured *in situ* using the WTW company instrument (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) or with a multiparametric Hydrolab, Transparency was measured using a Secchi disk. Major ions, main algal nutrients and chlorophyl a concentrations were determined following the standard methods (Vernon, 1960; Hekera, 1999; APHA, 2000).

3.3 Statistical analysis

Various multivariate statistical analyses were used in order to find the patterns of diatom distribution and factors influencing epiphytic diatom communities. Methods are described in detail in attached articles (Kollár et al., 2015; Letáková et al., 2016; Poulíčková et al., 2017).

4. RESULTS

The key results of the dissertation are included in the five attached articles. Recent knowledge about freshwater epiphytic diatoms has been summarized in paper I (Letáková et al., 2018 - see attached in the introduction). New LTM ESEM method, as the method allowing observation of diatom communities *in vivo* is the topic of paper II (Fránková et al., submitted). Substrate specificity and the spatial distribution of epiphytic diatoms in the lake Valagola were investigated in paper III (Letáková et al., 2016). Overall diversity of epiphytic diatom communities and the key factors of their distribution were studied in paper IV (Kollár et al., 2015). And finally finding the information about species complexes and their potential for biomonitoring was the target of paper V (Poulíčková et al., 2017).

4.1 Paper II

FRÁNKOVÁ M., POULÍČKOVÁ A., TIHLAŘÍKOVÁ E., NEDĚLA V., ŠUMBEROVÁ K. & LETÁKOVÁ M. (submitted): The low temperature method for Environmental Scanning Electron Microscopy – a new tool for observation of diatom assemblages *in vivo*. *Diatom Research.*

The Low Temperature Method for Environmental Scanning Electron Microscopy – a New Method for Observation of Diatom Assemblages *in vivo*

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Abstract

The Low Temperature method for sample stabilization in environmental scanning electron microscopy appears to be a promising tool for the observation of diatom assemblages *in vivo*. Use of the environmental scanning electron microscope, in comparison to the conventional scanning electron microscope, enables study of fresh material without any chemical pretreatment and conductive coating. The newly developed Low Temperature Method, introduced in this paper, offers higher resolution and better resistance of wet samples to radiation damage. We used natural epiphytic algal assemblages to image 3D structure of: i) biofilm/periphyton and its physical complexity, ii) diatoms with their extracellular mucilaginous secretions enabling cells to attach to the substrate, iii) diatom colony formation, and iv) intact diatom cells/frustules in contrast to separated empty valves observed in the conventional scanning electron microscope. This study demonstrates the potential of this new method for environmental scanning electron microscopy in diatom biology and ecology in comparison with other imaging methods.

Key words: *living diatoms, epiphyton, low temperature method for ESEM*

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Introduction

Diatoms are the most species-rich algal group represented by 75,000 described diatom taxa (Kociolek et al. 2018) and potentially many more species exist, as determined by extrapolation from an eclectic sample of genera and species complexes (Mann & Vanormelingen 2013) and the large number of unknown DNA sequences found in environmental samples (Adl et al. 2007). Diatoms are recognized as powerful bioindicators and used for water quality monitoring (Ibáñez et al. 2010, Rimet 2012).

Since the beginning of the 18th century when diatoms were first observed and described using a simple light microscope (LM), the technological advances in microscopy over the past 300 years have allowed the study of these amazing organisms in greater detail (Stoermer et al. 1964, Drum et al. 1966). The next period in the microscopic techniques development (20th century) is characterized by introduction of electron microscopy, at first Transmission electron microscope (TEM; Crawford 1974, von Stosch 1982) and later on Scanning electron microscope (SEM; Mann 1982, Cohn et al. 1989). TEM is useful mainly in algal cytology (organelles structure and organization) and contributed also to understanding of diatom frustule structure (Stoermer et al. 1964, Drum et al. 1966, Pickett-Heaps et al., 1979a, b; Edgar & Pickett-Heaps, 1984). SEM has provided amazing 3-D imagery of frustular morphology (Round et al. 19090), as well as the physiognomy of communities (Letáková et al. 2016). Unfortunately, most biological samples have a high-water content, thus observation in SEM requires several steps of pretreatment. Sample preparation for SEM involves fixing, freezing, dehydration and gold/platinum coating and some of these procedures may damage delicate samples (Joubert and Pillay 2008, Timp & Matsudaira 2008). However, preservation of delicate structures (e.g. mucilage) requires Critical Point Drying (Poulíčková et al. 2007) before SEM observation. Diatom taxonomy based on 3D frustule architecture went through significant progress, particularly after introduction of SEM techniques (Gerloff & Helmcke, 1974, Mann 1981). Nowadays SEM is a widely used tool and many new diatom taxa were erected based on diagnostic features recognizable in SEM only (Morales et al. 2001, Taylor et al. 2016, Ács et al. 2016). Although SEM techniques are irreplaceable in diatom taxonomy, their use in other fields (e.g. ecology) has certain limits. Primarily, we cannot observe intact fresh, native material such as shape and type of a colony or epiphyton directly *in situ* in great detail and with large depth of field.

The above-mentioned problems caused by sample preparation, can be overcome by using an environmental scanning electron microscope (ESEM). ESEM is an independent instrument and in majority of cases it is not a modification of conventional SEM, although there has been some attempt to modify a SEM for this purpose (Zetsche et al. 2016). The direct study of fully hydrated or electrically nonconductive dry samples in their native state, without the necessity of their surface covering by a conductive layer is possible due to the presence of high gas pressure, mostly water vapour in a specimen chamber of the ESEM (Donald 2003). The most important benefit of the ESEM is its capability of dynamical *in-situ* investigation of sample changes or reactions under influence of various conditions (Krausko et al. 2014). Moreover, detail sample description regarding diatom identification at the species level is possible (Richard et al. 2017).

Advantages of the ESEM for plant investigations have been already demonstrated (Popielarska-Konieczna et al. 2008, 2010, Stabentheiner et al. 2010, McGregor & Donald 2010, Vlašínová et al. 2017). The great potential of this method was also proved by introduction of the new method for study of small live mites (Tihlaříková et al. 2013). ESEM has also been used to visualize the microbial (particularly diatom) colonization of different types of substrates (sand grains, stones, artificial substrates; Joubert & Pillay 2008, Norbäck Ivarsson et al. 2013, Richard et al. 2017).

The ESEM observation of native aquatic samples is usually affected by radiation damage, whose effects can be reduced by working under low beam current and energy as well as by the use of special methods such as the Low Temperature Method (LTM) for ESEM (Neděla et al. 2012). Ideally, these two ESEM parameters are applied in combination, along with advanced low noise detectors with very high detection efficiency (Neděla et al. 2011). The LTM for ESEM is a method pushing the limits in electron beam observation of untreated fresh samples with higher resolution and under lowered impact of electron beam radiation. The method is based on low temperature stabilization of a sample using mutual combination of optimized speeds of gas pumping and sample cooling and is generally applicable in ESEM. On the other hand, if environmental humidity in ESEM is decreased, this method can be also used for gentle sample drying and preparation for repetitive high-resolution observation in SEM.

The purpose of the present study is to present a summary of basic principles of the new LTM for ESEM described by Neděla et al. (2015). The potential application to microalgal, particularly fresh diatom material is demonstrated on epiphytic assemblages.

Material and methods

Fresh diatom material origin

Epiphyton samples were collected with the whole substrate at two eutrophic fishponds in June 2016. The localities are namely the Dehtář fishpond (49°0'26"N, 14°17'36"E) and the Žebětínský fishpond (49°12'50"N, 16°29'32"E) in the Czech Republic. The host plants investigated were common aquatic macrophytes *Lemna gibba* L., *Phalaris arundinacea* L. and *Phragmites australis* (Cav.) Steud. The whole plants (*Lemna gibba*) or plant stalks (10 cm long sections) were tacked to a polystyrene matrix with paper pins, placed into plastic containers containing water from the locality and transported to the laboratory.

Observation of the samples using the LTM for ESEM

Fresh algal assemblages were observed on a stem epidermis (*Phalaris arundinacea*, *Phragmites australis*) and roots (*Lemna gibba*) using the LTM for ESEM without any pretreatment. Samples were observed using the FEI Peltier stage equipped with a special flat cylindrical brass sample holder. Samples were cut to 4–8 mm^2 (in the case of *Lemna gibba* smaller) segments and placed into a drop of 2 μl of water, to obtain better thermal contact between the sample and the Peltier stage. The conditions for observation were adjusted as follows: the air pressure was 250 Pa, sample temperature –20 °C (Neděla et al. 2015). All observations were performed on FEI ESEM QUANTA 650FEG with beam energy 20 kV, probe current 35 pA and working distance 8.5 mm.

Results

Observation of epiphyton complexity (Figs 1–2)

A mixture of organisms constituting epiphytic assemblages on *Phalaris arundinacea* is presented in figure 1. Pennate diatoms are attached to the substrate (Figs 1 c, e) within the biofilm. We can also recognize some planktonic microalgae present, due to their sedimentation from the water column, including *Parapediastrum biradiatum* (Meyen) E. Hegewald (Fig. 1 a), *Microcystis* Lemmermann (Fig. 1 b), *Desmodesmus* (Chodat) S.S.An, T. Friedl & E. Hegewald (Fig. 1 d) and *Puncticulata balatonis* (Pantocsek) Wojtal & Budzyńska (Fig. l f).

Other planktonic microalgae are clearly visible in Fig. 2, including *Monactinus simplex* (Meyen) Corda (Fig. 2 a) and intact colonies of the diatom species *Aulacoseira ambigua* (Grunow) Simonsen (Fig. 2 b).

Observation of extra cellular mucilaginous secretions (Figs 3–4)

The rhizodermis of *Lemna gibba* is colonized by diatoms producing extracellular mucilaginous secretions (e.g. pads, stalks). Some genera are attached by their valve face (*Cocconeis* Ehrenberg, Fig. 3 b), while others are attached by one end (*Gomphonema* Ehrenberg Fig. 3 a, 4 a, *Fragilaria* Lyngbye Fig. 4 c). Stalks are clearly visible in the case of *Gomphonema* (Fig. 4 a). Fine filaments evident (Fig. 4 b) are hyphae of saprotrophic fungi (Vohník, pers. comm.).

Observation of colony formation (Figs 2, 6)

Both, pennate and centric diatoms create typical colonies, which can be observed in natural form using the LTM for ESEM. The centric diatom *Aulacoseira ambigua* creates spiral coiled colonies (Fig. 2 b). *Fragilaria* (Fig. 6) cells are attached to each other by valve face and mucilaginous connections are clearly visible (arrow).

Observation of details of the whole intact diatom cell (Fig. 7)

Figure 7 shows external the valve surface of *Navicula radiosa* Kützing, including the raphe. The image shows enough details important for species identification and the result is almost comparable with classic SEM.

Discussion

Diatom taxonomy is traditionally based on silica frustule architecture and requires detailed analysis of many fine structures. Although, molecular methods are increasingly used in the last decade (Zimmermann et al. 2014), microscopy remains a principal tool for species identification and new taxa description (Poulíčková et al. 2016). Classic diatom microscopic observations are made only after extensive preparation, including cleaning in strong acids or oxidation agents (mixture of nitric and sulphuric acids, hydrogen peroxide) and subsequent mounting to permanent slides in the case of LM, or conductive coating (using gold or platinum) in the case of SEM. Small depth of field is the most serious limitation of classical light microscopy (LM). This disadvantage of LM should be eliminated using a complementary technique to traditional LM – holographic microscopy (HM, Gabor 1948, Almeida et al. 1971) and digital holographic microscopy (DHM). Holographic microscopy enables ten- to a hundred times greater depth of focus than LM (Zetsche et al. 2016). These authors showed that DHM enables not only visualization of internal frustule structures, but also transparent excretions of diatoms – extracellular polysaccharide substances (EPS). DHM is a versatile technique allowing investigation "*in vivo*" without staining, however it still works at magnification scales comparable to LM (Zetsche et al. 2016).

Thin and hollow cleaned diatoms were used for testing many imaging techniques (Bertilson et al. 2009, Piper 2011). High-resolution computed tomography reconstructs the frustule inner structure from a set of projections. Reconstructed tomograms can be visualized using software on standard personal computers (Bertilson et al. 2009). During the procedure, the diatom is mounted at the fine tip of a glass capillary placed in a rotatable holder. The tomogram resolution is limited by the number of projections, i.e. more projections bring higher resolution (Bertilson et al. 2009). Similar principles are used in x-ray imaging laminography, where a sample is placed on a membrane, which simplifies the sample preparation (Hoshino et al. 2011). We should mention also confocal laser scanning microscopy (CLSM) and methods combining data from CLSM and SEM. Indeed, they generate exact 3D models of diatom frustules (Friedrichs et al. 2012). For imaging using this technology, diatoms should be free of organic content, particularly the frustulin membrane surrounding silica frustule (Poll et al. 1999).

ESEM seems to be highly promising for diatom ecological research, due to resolution higher than in LM and easier and less destructive sample preparation than in the case of classic SEM. Moreover, it is much cheaper than Cryo-SEM (Neděla et al. 2014, 2016). However, it cannot replace standard SEM in diatom taxonomy. LTM for ESEM represents a methodological improvement with an even better stability of wet samples and higher resolution than for a suitable pressure at the temperature closely above 0°C, as it is usual in ESEM observation. Observed samples in commonly used ESEM are mostly covered by a thin layer of water (Fig. 5) ensure hydration of sample surface. LTM for ESEM is based on a suitable sample chamber pumping procedure in combination with sample cooling - with a defined temperature gradient. These conditions lead to a gentle evaporation/sublimation of the surface water layer covering the sample. The sample is stabilized at a temperature around -20 °C and pressure around 150 Pa, and it is still observed with 100 % of moisture. A clean sample surface (without ice or water) enables higher resolution (Figs 1-4, 6,7). Note that the described method is different from the method previously used for biofilms and it is referred to as the Low-temperature SEM (Decho 2000). LT SEM is based on deep sample freezing (from -100 to -170°C) under the conditions at the sample chamber pressure lower than 0.01 Pa and observation in conventional SEM. It means that samples in this case are not native, but frozen.

LTM for ESEM brings advantages of fresh material observation, which is particularly useful in the case of attached microalgae and diatoms. Density and distribution of epiphytes on plant surfaces are of interest in substrate specificity studies (Mutinová et al. 2016, Letáková et al. 2016, Fránková et al. 2017), where reasons for differences between host plants are discussed. Better knowledge about diatom/microalgae adaptations to sessile life can bring new insight into their autecology (light, nutrient requirements).

Benthic diatoms are classified into functional groups, e.g. ecological guilds (Passy 2007, Rimet and Bouchez 2012a,b, Fránková et al. 2017) or eco-morphological functional groups (B-Béres et al. 2016) which reflect relationships between taxa and environmental factors (B-Béres et al. 2016). However, all standard sampling methods (for LM and SEM) are based on substrate scraping (for instance using a tooth brush or a scalpel) which lead to destruction of the periphyton structure (Kelly et al. 1998). Diatoms are characterized by formation of different types of colonies, which can be species specific. ESEM enables us to study colony formation in great details – how cells are stick together using extracellular mucilaginous secretions (Fig. 6).

Moreover, there exist other natural diatom cell associations which should be imaged without disturbances, particularly during sexual reproduction. Briefly, compatible sexualized cells freely moving on the substrate are pairing valve-valve or girdle-girdle creating pairs or triplets. Gametes fusion is followed by zygote formation and expansion which is called auxosporulation. During this process auxospores produce very fine, slightly silicified structures and envelopes such as incunabula and perizonia. Although the silicified structures should be studied in details using SEM (Mann & Poulíčková 2009), most gentle components (mucilage capsules, specific position of cells) are lost during preparation. ESEM should be ideal method to observe these processes *in vivo*.

Moreover, mucilaginous material is produced by diatoms also during sexual reproduction (Poulíčková et al. 2007). Such mucilaginous envelopes protecting gametangia were observed in LM using India ink staining and in SEM fixation, dehydration through an ethanol series followed by drying in a critical point dryer, which are time consuming procedures (Poulíčková et al. 2007). ESEM seems to be a suitable method for imaging such protective envelopes.

In summary, fresh material in ESEM is observed under thermodynamic equilibrium in 100% relative humidity. Usually, these conditions are maintained slightly above 0°C when the sample surface is covered with thin layer of liquid water. The thickness of this layer can be reduced by decreasing relative humidity, however the fine surface microstructure details can be still invisible (Fig. 8). This method is useful for study of liquids or samples sensitive for cooling. The LTM for ESEM was optimized for using under reduced sample temperature below 0°C. The clear liquid water on the sample surface is gently evaporated whereas the liquid solution stays inside the sample due to its different partial pressure. Thank to this procedure the sample is stabilized, hydrated and free of ice crystals. The soft sample morphology can be observed with higher resolution (Fig. 9) and specific structures like mucilaginous material (Fig. 3, 4 – stalks of *Gomphonema* and Fig. 6 white arrow – mucilaginous connection within a *Fragilaria* colony) are visible.

Conclusion

The paper shows applicability of the LTM for ESEM for observation of aquatic plants with epiphytic microalgae (particularly diatoms). In comparison with standard ESEM methods, LTM achieves higher resolution and better sample stability by reducing sample temperature and gas pressure, while the humidity is kept almost 100 %.

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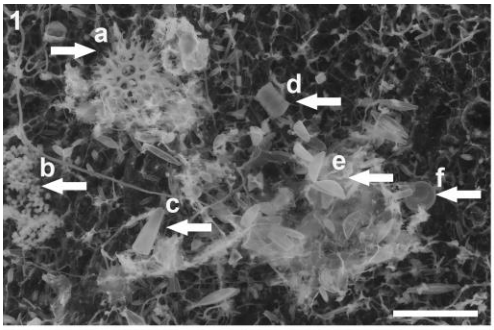
Figs. 1–4, 6. Epiphyton samples from the locality Dehtář imaged by LTM ESEM. **Fig. 5.** Epiphyton from the locality Žebětín imaged in wet state by commonly used ESEM (without LTM). **Fig. 7.** Epiphyton from the locality Žebětín imaged by LTM ESEM. **Scale bars: Figs 1–3**. 50 µm, **Figs 4, 6.** 20 µm, **Fig. 5.** 10 µm and **Fig. 7.** 3 µm. **Fig. 1.** heterogeneous assemblage of cyanobacteria and algae on epidermis of *Phalaris arundinacea*, a – *Parapediastrum biradiatum*, b – *Microcystis* sp., c – *Gomphonema* sp., d – *Desmodesmus* sp., e – *Cymbella* sensu lato, f – *Puncticulata balatonis*. **Fig. 2**. epiphyton, sedimented planctic species, a – *Monactinus simplex,* b – *Aulacoseira ambigua*. **Fig. 3**. rhizodermis of *Lemna gibba*, a – *Gomphonema* sp. attached by a stalk, b – *Cocconeis* sp. attached by valve face, c – *Ulnaria ulna* **Fig. 4.** rhizodermis of *Lemna gibba,* a – stalks of *Gomphonema* sp., b – hyphae of saprotrophic fungi, c – *Fragilaria* sp. **Fig. 5.** *Achnanthidium minutissimum* on *Phragmites australis* in commonly used ESEM without our methodological improvement LTM **Fig. 6.** *Fragilaria* sp., mucilaginous connection between cells in a colony (arrow). **Fig. 7.** epidermis of *Phragmites australis*, detail external view of *Navicula radiosa*.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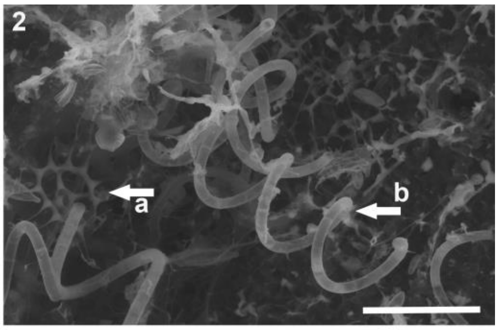
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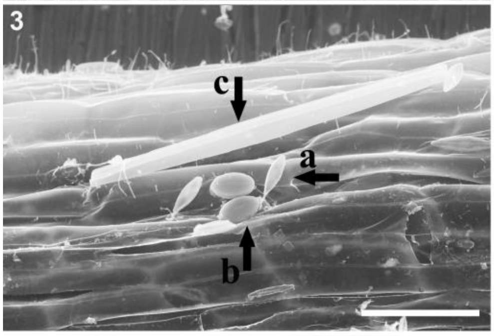
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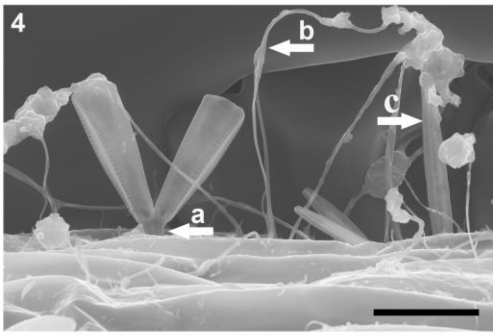
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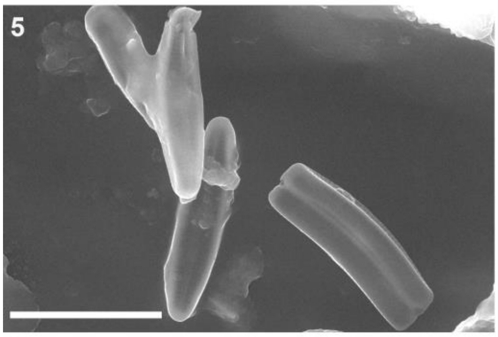
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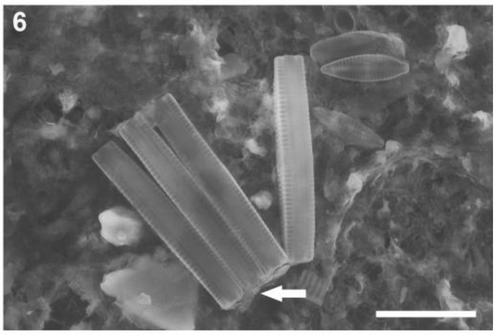
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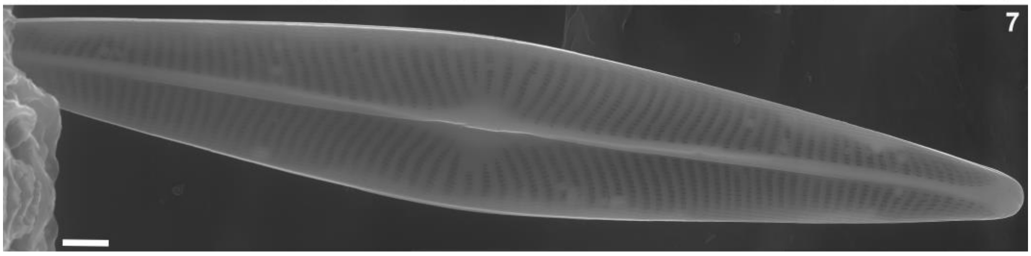
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4.2 Paper III

LETÁKOVÁ M., CANTONATI M., HAŠLER P., ANGELI N., & POULÍČKOVÁ A. (2016): Substrate specificity and fine-scale distribution of epiphytic diatoms in a shallow tarn in the Brenta Dolomites (South-eastern Alps). *Plant Ecology and Evolution* 149 (2): 144-156.

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REGULAR PAPER

Substrate specificity and fine-scale distribution of epiphytic diatoms in a shallow tarn in the Brenta Dolomites (south-eastern Alps)

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Background and aims – The host-specificity of epiphytic diatom species has long been debated. Scuba divers sampled epiphytic diatoms in the shallow Alpine Lake Valagola (average depth c. 2 m) along seven transects (length: 30–144 m) in West-East direction. The bottom of the tarn was covered by macrophytes dominated by *Chara aspera* and *Potamogeton gramineus*. Factors affecting epiphytic-diatom spatial distribution at a fine scale were tested.
Methods – Dataset was tested using Redundancy Analysis (CANOCO package) and one-way ANOVA (NCSS package).
Key results – The analysis separated sampling sites into two groups: the tarn shore dominated by *Potamogeton gramineus*, and the central area dominated by *Chara aspera*. Diatom species richness, diversity, and composition differed significantly between the two main host plants. *Potamogeton gramineus* assemblages were characterized by higher species richness and diversity, and by the large-celled, adnate diatom species *Epithemia adnata*, *Rhopalodia gibba*, *Eunotia arcus* and *E. arcubus*. *Chara aspera* was preferred by the small-celled, motile diatom species *Brachysira neoexilis* and *Encyonopsis cesatii*.
Conclusions – The spatial distribution of epiphytic diatoms in the shallow, oligo-mesotrophic Lake Valagola is influenced by host plant composition and distribution. Epiphyton size structure suggests that *Chara* represents a less appropriate substrate for long diatoms.

Key words – Epiphyton, mountain lake, host specificity, epiphyton size structure, *Chara aspera*, *Potamogeton gramineus*, diatoms.

INTRODUCTION

While lake environments are separated in habitats, zones, and gradients (Poulíčková et al. 2008, 2014), microphototrophs are typically classified as benthic and planktic. Both categories perform a range of ecosystem functions and contribute significantly to lake biodiversity. However, photoautotrophs that inhabit benthic environments have received less attention than the phytoplankton (Cantonati & Lowe 2014, Poulíčková et al. 2014). Aquatic macrophytes are key components in spatial heterogeneity (Thomaz et al. 2008). Epiphytic microalgae living in association with macroalgae and aquatic macrophytes contribute significantly to the primary production of lakes, particularly in the littoral zone (Cattaneo & Kalff 1980, Vander Zanden et al. 2006, Cano et al. 2008). Epiphyton is an important source of food for invertebrates (Cattaneo 1983), and has been proposed as a target community for the assessment of lake trophic status (Lalonde & Downing 1991, Poulíčková et al. 2004).

Epiphytic algae are challenging to be studied quantitatively because they are difficult to separate from their substrate, and because their spatial distribution is heterogeneous and not fully understood. Diatoms, cyanobacteria, and green algae are the most common benthic microalgae (Poulíčková et al. 2014), comprising the majority of the epiphyton biomass (Pomazkina et al. 2012, Neif et al. 2013). Diatoms are represented by motile species gliding on various substrates, and species attached mostly via mucilagenous structures.

Epiphyton species composition on submerged macrophytes differs between lakes (Kiss et al. 2003) and phosphorus has been shown to have a significant influence (Cattaneo & Kalff 1980, Fairchild et al. 1985). Lake trophic status can influence phytoplankton abundance (and consequently light availability at the bottom), biomass of submerged plants (substrate availability), and subsequent vertical distribution of epiphyton (Lalonde & Downing 1991, Romo et al. 2007). Substrate specificity, which in real ecosystems is combined with other possible influencing factors (physical, chemical, biological), represents a multivariate problem. Thus previous studies were mostly observational (Cattaneo & Kalff 1980, Millie & Lowe 1983, Lalonde & Downing 1991, Potapova & Charles 2005, Cantonati et al. 2012).

This study aims to analyse the detailed spatial distribution of epiphytic diatom assemblages in the shallow Alpine Lake Valagola. We tested differences in epiphyte composition and distribution on a single sampling date: (1) between the marginal and central part of the lake, and (2) between different macrophytes.

MATERIAL AND METHODS

Study site

Lake Valagola (46°9′56,462″N 10°49′13,980″E) is located in the western part of the Brenta Dolomites (south-eastern Alps, Adamello-Brenta Nature Park, Italy), at an elevation of 1,595 m a.s.l. The lake was formed by a Daun stadial moraine (Trevisan 1939). The lake does not have direct inlets. The Valagola stream, flowing down the Nardis Valley and collecting the meltwaters of the small Agola and Prato-Fiorito glaciers, disappears into the alluvial fan formed by the stream itself slightly upstream of the tarn. Since the average discharge of the Valagola stream is about four times that of the tarn outlet, it must be assumed that its waters reach the groundwater through the fan deposits, and part of this water re-emerges from the bottom of the tarn feeding it, which would be consistent with the tectonic and carbonate context. The lake level can consequently undergo important fluctuations, almost reaching the height of the small forest-operations road bordering the eastern bank and the hiking path during very-rainy periods. The morainic rim is permeable, and the depths reached by the lake today are possible

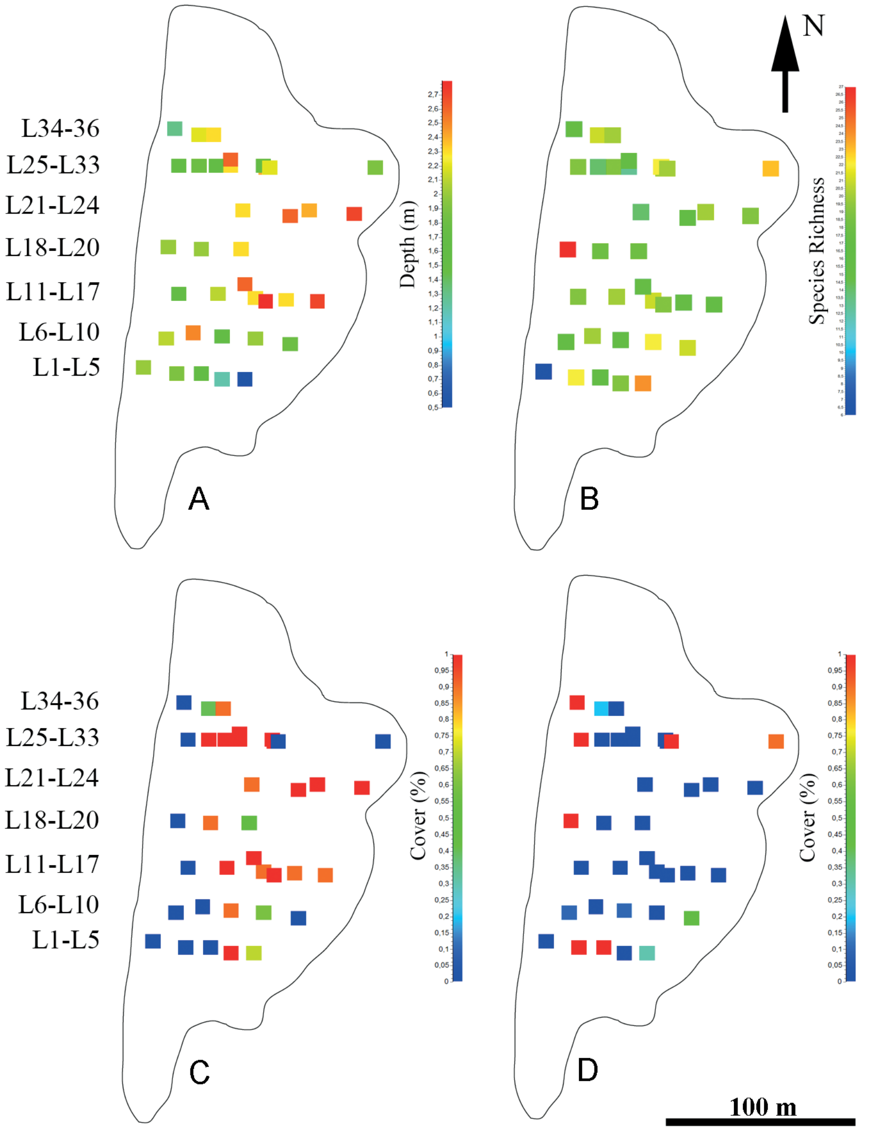

Figure 1 – Location of 36 sampling sites (L1–L36; for details see table 3) within seven transects in West-East direction and their characteristics as follows: A, sampling site depths; B, epiphytic diatom species richness; C, *Chara aspera* cover; D, *Potamogeton gramineus* cover.

Table 1 – **Hydrochemical analysis.**
Average from nine measurements (9 September, 16 October, 13 November, 16 December 2013; 19 March, 19 June, 25 July, 3 September, 14 October 2014) and minimum and maximum value.

Depth (m)	Temperature (°C)	Conductivity (µS cm^{-1})	pH	Redox
0	8.0 (0.2–13.8)	239 (187–305)	8.2 (7.9–8.7)	264 (208–320)
0.5	10.9 (3.3–18.2)	222 (159–295)	8.2 (7.8–8.8)	247 (208–320)
1	10.9 (3.3–17.9)	222 (160–295)	8.3 (7.9–8.8)	249 (207–320)
1.5	9.7 (3.3–14.8)	232 (187–296)	8.2 (8.0–8.6)	248 (206–320)
2	10.7 (3.4–17.7)	222 (159–296)	8.3 (8.1–8.8)	249 (206–321)
2.5	10.6 (4.1–17.7)	240 (195–355)	8.3 (8.1–8.6)	250 (206–321)

Table 2 – **Hydrochemical analysis**.
Data measured on 11 September 2013 and 3 September 2014. *measurement only on 11 September 2013, ** measurement only on 3 September 2014.

Depth (m)	Alkalinity (mg/l)	TN (µg/l)	N-NO$_3^-$ (µg/l)	N-NH$_4^+$ (µg/l)	P-PO$_4$ (µg/l)	TP (µg/l)	SiO$_2$ (mg/l)	SO$_4^{2-}$ (mg/l)	F (µg/l)	Chl-*a* (mg/l)
0.5	81–116	427–407	190*	12–39	1–1	14–12	2.2–0.5	1.4*	68*	0.002**
1.5	80–116	401–403	187*	13–38	1–1	15–14	2.2–0.5	1.5*	54*	0.001**
2.5	81–116	341–408	190*	12–70	1–1	17–16	2.3–0.5	1.3*	89*	0.001**

only thanks to an artificial rim, which was completed in the 1970s. Lake Valagola might thus have been characterized by a very long period of fluvio-lacustrine regime during its post-glacial history.

Lake Valagola is a shallow tarn with depths ranging from 1.6 to 2.7 m in the western portion and from 0.5 to 2.8 m in the deeper eastern part (fig. 1A). From the thermal point of view, Lake Valagola is a cold polymictic lake (table 1) with average surface temperature 8°C. Snow and ice cover last 5–6 months. Conductivity and slightly alkaline pH are consistent with the carbonate lithology of the area. The values of the main algal nutrients (table 2) measured during the present investigation (in particular nitrates and total phosphorus) do not differ significantly from the few hydrochemical data available in the literature (ISMA 1997). Nitrate values even appear to be lower today. A meso-oligotrophic status can thus be confirmed. This condition is favored by its shallowness, and it is mainly determined by the nearby cattle barn with pastures reaching the shores of the lake. Phytoplankton abundance is low (average Chl-*a* is 1.3 µg l^{-1}) with diatoms, Cryptophyceae, Chrysophyceae, and Dinophyceae being the numerically best represented groups (ISMA 1997). Bentic assemblages are well developed during the ice-free period and dominated by Zygnematophyceae (*Spirogyra*), diatoms and cyanobacteria (data not shown).

Sampling

Macrophytes were sampled with the assistance of Scuba divers on 10 Sep. 2013. Seven transects (length: 30–144 m) in c. West-East direction (i.e. perpendicular to the maximum dimension of the lake) were marked by strong ropes firmly assured to the opposing shores (table 3). A boat moved along the ropes and distance from the shore (with a measuring tape ribbon), GPS position, and depth (with an echosounder) of each site (five sites per each transect, L1–L36, table 3) were measured. Samples of epiphytic algae together with their substrate were obtained by collecting the upper 10–20 cm of macrophytes (enough plant material to fill a large transparent polyethylene bag) growing on the bottom at sites mentioned above (L1–L36). Samples close to lake banks belong to the group "marginal part of the lake" (sites L1, 5, 6, 10, 11, 15, 16, 20, 21, 25, 26, 30, 31, 36). Subsamples (100 ml sampling bottles) for the study of epiphytic diatoms were taken and fixed with formaldehyde (2–4% final conc.). Samples and slides are kept in the Museo delle Scienze – MUSE, Trento diatom collection under numbers cLIM005 DIAT 2316-2351. The rest of plant material served for identification of macrophytes and a selection will be deposited in the herbarium of the Museo delle Scienze – MUSE (TR). General limnological characterization of the site was based on measurements of ecological variables in 2013–2014 and single sampling of epilithon, epipelon, and plankton taken from the southern shore (close to L1) on 10 Sep. 2013. Phytoplankton was sampled qualitatively with a 10-µm mesh net towed by a boat operated as to describe a sinusoidal trajectory.

During 2013–2014, physical and chemical factors (temperature, conductivity, pH, redox) were measured with a multiparametric Hydrolab probe (2013: 9 September, 16 October, 13 November, 16 December; 2014: 19 March, 19 June, 25 July, 3 September, 14 October). On 19 Mar. 2014 no operations could be carried out on the water column, since the lake was covered by ice and snow (several layers). In September 2013 and 2014, complete (major ions and algal nutrients)

Table 3 – Basic characteristics and host plants of sampling points.

Sampling point	Transect	Depth(m)	Latitude WGS84_N	Longitude WGS84_E	*Calliergon giganteum*	*Chara aspera*	*Chara virgata*	*Potamogeton gramineus*
L1	1	2	4609894	1049180	0	0	0	0
L2	1	1.9	4609891	1049196	0	0	0	1
L3	1	1.6	4609891	1049208	0	0	0	1
L4	1	1.2	4609888	1049218	0	1	0	0
L5	1	0.5	4609888	1049229	0	0.7	0	0.3
L6	2	1.8	4609906	1049251	0	0	0.5	0.5
L7	2	2	4609909	1049234	0	0.6	0.4	0
L8	2	1.6	4609910	1049218	0	0.9	0	0.1
L9	2	2.5	4609912	1049204	0	0	0	0
L10	2	2.1	4609909	1049191	0	0	0.9	0.1
L11	3	1.6	4609932	1049197	0	0	0	0
L12	3	2.1	4609932	1049216	0	1	0	0
L13	3	2.3	4609930	1049234	0	0.9	0.1	0
L14	3	2.3	4609929	1049249	0	0.9	0.1	0
L15	3	2.7	4609928	1049264	0	0.9	0.1	0
L16	4	2	4609956	1049192	0	0	0	1
L17	4	2	4609955	1049208	0	0.9	0.1	0
L18	4	2.3	4609955	1049227	0	0.5	0.5	0
L19	4	2.6	4609937	1049229	0	1	0	0
L20	4	2.8	4609928	1049239	0	1	0	0
L21	5	1.9	4609977	1049292	0.1	0	0	0.9
L22	5	2.7	4609973	1049282	0	1	0	0
L23	5	2.6	4609972	1049251	0	1	0	0
L24	5	2.3	4609975	1049228	0	0.9	0.1	0
L25	5	2.4	4609975	1049200	0	1	0	0
L26	6	1.6	4609998	1049197	0	0	0	1
L27	6	1.8	4609998	1049207	0	1	0	0
L28	6	1.6	4609998	1049215	0	1	0	0
L29	6	2.3	4609998	1049222	0	1	0	0
L30	6	2.4	4609997	1049239	0	1	0	0
L31	7	1.3	4610017	1049195	0	0	0	1
L32	7	2.2	4610014	1049207	0	0.4	0.4	0.2
L33	7	2.3	4610014	1049214	0	0.9	0.1	0
L34	7	2.6	4610001	1049222	0	1	0	0
L35	7	1.7	4609998	1049238	0	1	0	0
L36	7	2.2	4609997	1049241	0	0	0	1

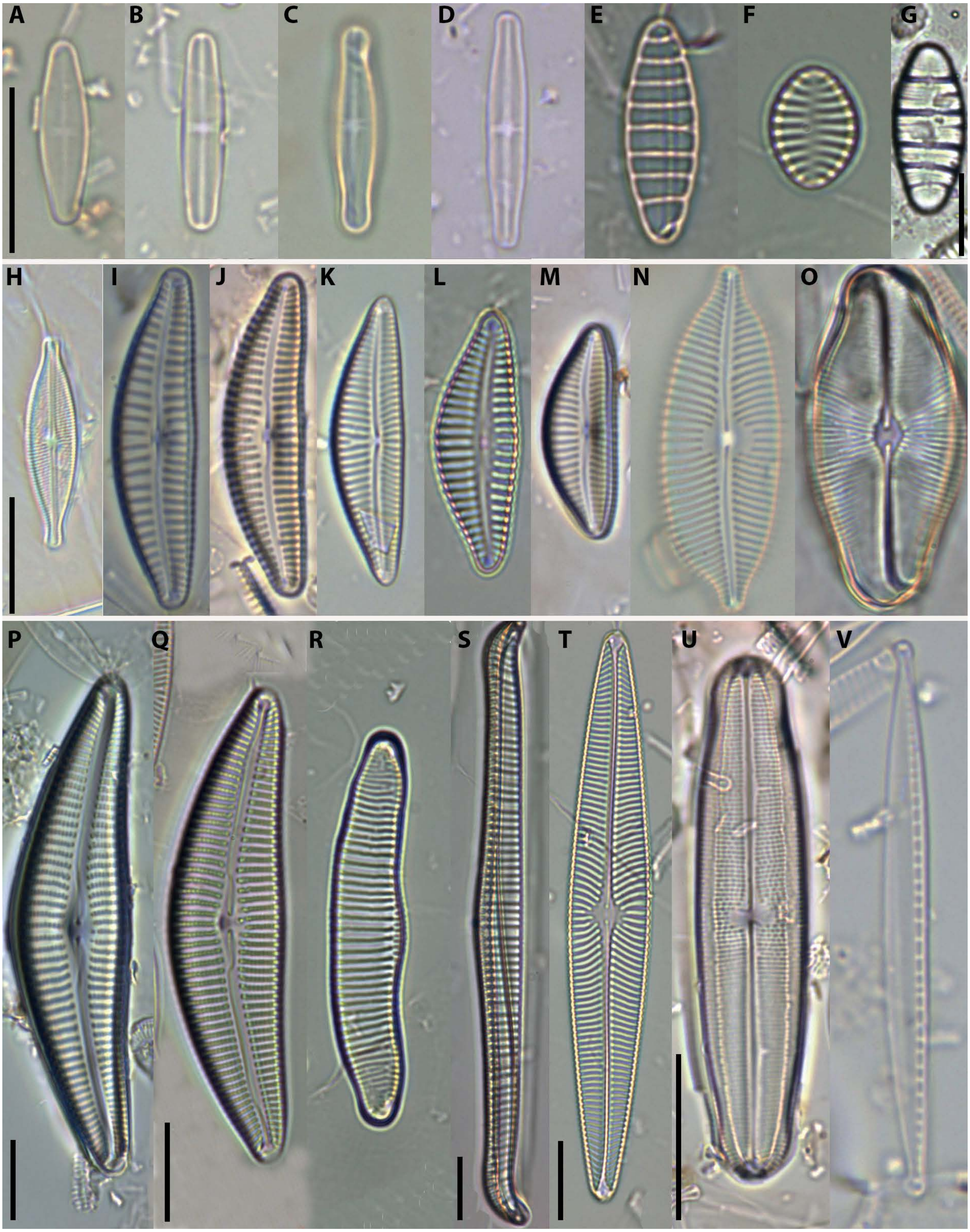

Figure 2 – Selected species: A, *Achnanthidium dolomiticum* M.Cantonati & Lange-Bert.; B, *Achnanthidium lineare* W.Sm.; C, *Achnanthidium minutissimum* var. *jackii* (Rabenh.) Lange-Bert.; D, *Achnanthidium minutissimum* var. *minutissimum*; E, *Denticula tenuis* Kütz.; F, *Staurosira venter* (Ehrenb.) Cleve & J.D.Moeller; G, *Diatoma mesodon* (Ehrenb.) Kütz.; H, *Brachysira neoexilis*; I, *Cymbella excisiformis* Krammer; J, *C. parva* (W.Sm.) Kirchn.; K, *C. levis* Nägeli; L, *Cymbella subleptoceros*; M, *Cymbella* cf. *hustedtii* var. *rhombica* Krammer; N, *Cymbopleura frequens* Krammer; O, *Eucocconeis flexella* (Kütz.) F.Meister; P, *Cymbella cymbiformis* C.Agardh; Q, *Cymbella scutariana* Krammer; R, *Eunotia soleirolii* (Kütz.) Rabenh.; S, *Rhopalodia parallela* (Grunow) O.Müll.; T, *Navicula radiosa* Kütz.; U, *Neidium affine* (Ehrenb.) Pfitzer; V, *Nitzschia oligotraphenta* (Lange-Bert.) Lange-Bert. Scale bar = 10μm.

analyses were carried out at the environmental chemistry lab of the E. Mach Foundation following standard methods (APHA 2000).

Diatom preparation

Samples were treated with 30% H_2O_2 for 24 hours. The Erlenmayer flasks with treated samples were subsequently heated up to a boiling point and kept boiling approximately for 60 minutes. Small amounts of $K_2Cr_2O_7$ and c. 500 µl, 37% HCl were added into the hot samples. Samples were centrifuged and cleaned with distilled water until reaching neutrality. Cleaned diatom frustules were mounted in Naphrax. At least 400 valves were counted and percentage of relative abundance for every of 36 slides were evaluated together with species level identification using the following literature: Krammer (2000, 2002, 2003), Lange-Bertalot (2001), Levkov (2009), Lange-Bertalot et al. (2011), and Hofmann et al. (2013). Nomenclature was harmonized using AlgaeBase (Guiry & Guiry 2015). Slides were observed using light microscope Zeiss Axioskop 2 (Zeiss, Jena, Germany) equipped with phase-contrast and with an Axiocam digital camera. Two permanent slides representing samples of epiphyton exclusively from *Chara/Potamogeton* (N. DIAT 2327, DIAT 2317) were used for diatom cell size measurements (200 cells were measured for each substrate). SEM images were taken from herbarized (dried) material from the stations 4.4 and 7.1, corresponding to epiphytic-diatom samples cLIM005 DIAT 2334 (for *Chara aspera* Willd. 100%) and cLIM005 DIAT 2346 (for *Potamogeton gramineus* L. 100%) respectively, using a Scanning Electron Microscope Zeiss EVO 40 XVP Zeiss after gold coating.

Statistical analysis

The statistical package Canoco for Windows 4.5 (ter Braak & Šmilauer 2002) was used to test relationship among diatom assemblages and host plants. Species data were transformed using the Hellinger transformation before carrying out multivariate analyses. Detrended Correspondence Analysis (DCA) based on detrending by segments showed short gradients on first (2.509) and second axis (1.137). Redundancy Analysis (RDA) was carried out as follows: *Chara aspera* and *Potamogeton gramineus* as the most frequent host plants in the Lago di Valagola were set as environmental data, depth as covariable, and position in the lake as supplementary variable. Scaling method was focused on inter-species correlation. Both automatic and manual forward selection of environmental variables (Monte Carlo Permutation test, 499 unrestricted permutations) was used to test statistical significance of species-environmental variables relationship. Both *Chara aspera* and *Potamogeton gramineus* showed low inflation factor ($VIF_{Chaspe} = 2.602$, $VIF_{Potgram} = 2.674$). Visualisation, T-values biplot statistics and Shannon diversity index calculation were processed by CanoDraw for Windows 4.0. Differences between averages of diatom length, width, length/width ratio and Shannon diversity index between *Chara aspera* and *Potamogeton gramineus* were tested statistically using One Way ANOVA (NCSS, Hintze 2006).

Table 4 – Results of Redundancy Analysis.
Lambda1, variable explanation for Conditional Effects (%); LambdaA, variable explanation for Marginal Effects (%); P, significance of F statistics; F, result of F statistics; Chara, *Chara aspera*; Potgram, *Potamogeton gramineus*.

Marginal Effects

Variable	Lambda1	p	F
Chara	0.17	0.002	7.13
Potgram	0.11	0.006	4.35

Conditional Effects

Variable	LambdaA	p	F
Chara	0.17	0.002	7.13
Potgram	0.06	0.008	3.05

RESULTS

The majority of the tarn bottom was overgrown with the stonewort *Chara aspera*, with scattered populations of *C. virgata* Kütz. The lake shore in the western portion was covered by a narrow belt of *Potamogeton gramineus*. A small population of the bryophyte *Calliergon giganteum* (Schimp.) Kindb. grew only in one site (L21) located on the western shore. Host (substrate) plant composition of each sample is given in table 3 and co-dominating host plant's relative representation is shown in fig. 1C & D.

A total of 78 epiphytic-diatom species (some of them are documented in fig. 2) were identified (electronic appendix). Species richness of the individual epiphyton samples ranged from 11 to 38 taxa. In comparison, single samples of epilithon and epipelon (close to site L1) included 28 and 30 species respectively. The vast majority of species found on the macrophytes were benthic pennate, while the percentage of centric diatoms was < 36% (*Cyclotella* sp.). The most frequent epiphytic diatoms were *Pseudostaurosira polonica* (M.Witak & Lange-Bert.) E.Morales & Edlund, with relative representation ranging from 1 to 74%, *Staurosirella pinnata* (Ehrenb.) D.M.Williams & Round, with proportions up to 27%, and *Encyonopsis subminuta* Krammer & E.Reichardt, with relative abundances up to 25%. In comparison, epilithon and epipelon (site L1), were dominated by *Pseudostaurosira polonica* and *Achnanthidium straubianum* (Lange-Bert.) Lange-Bert. Phytoplankton was represented by *Cyclotella* cf. *radiosa* (Grunow) Lemmerm.

Redundancy Analysis (table 4) showed statistically significant relationships between species composition and environmental variables (F = 7.024, p = 0.004). The first ordination axis explains 18.0% and the second 6.9% of species data variation. The analysis separated sampling sites into two basic groups: (i) sites dominated by *Chara aspera* (central part of the lake) and (ii) sites dominated by *Potamogeton gramineus* (marginal parts of the lake), host plants at L1, L9 and L11 were not identified (fig. 3). Monte Carlo permutation test showed a statistically significant effect of *Chara aspera* on distribution of epiphytic diatoms (conditional effect: F = 7.13, p = 0.002; Marginal effect: F = 7.13, p = 0.002).

The influence of *Potamogeton gramineus* on diatom distribution was also significant (conditional effects: F = 3.05, p = 0.008, marginal effects F = 4.35, p = 0.006).

Diatom assemblages of both main groups differed in species richness and diversity. A significant difference (F = 9.01, p = 0.0053) in diatom distribution (expressed as Shannon index; fig. 4) was found between *C. aspera* (2.33±0.21) and *P. gramineus* (2.57±0.19). Diatom taxa positively correlated with *C. aspera* (fig. 5) included: *Brachysira neoexilis* Lange-Bert., *Cyclotella* sp., *Encyonopsis cesatii* (Rabenh.) Krammer (zone 1 in fig. 5). Diatoms positively correlated with *P. gramineus* included *Epithemia adnata* (Kütz.) Bréb., *Eunotia arcubus* Nörpel & Lange-Bert., *Eunotia arcus* Ehrenb., *Rhopalodia gibba* (Ehrenb.) O.Mül., *Cymbella subleptoceros* Krammer (zone 3 in fig. 5). Zone 2 in fig. 5 contains species present on both plants, e.g. the euryvalent species complex *Achnanthidium minutissimum* (Kütz.) Czarn. Observations on herbarized material showed, that *Potamogeton* was colonized by higher number of diatom cells than *Chara*, moreover attached diatoms usually comprised large colonies on *Potamogeton* (fig. 6E–G). Single diatom cells were able to colonize sporadically *Chara* thalli (fig. 6A–D). Diatom size measurements exclusively from *Potamogeton/Chara* samples showed that significantly longer diatoms are present on the surface of *Potamogeton* than on *Chara* (length F = 11.49, p = 0.0008, length/width ratio F = 12.39, p= 0.0005, fig. 7). These results are in congruence with species composition typical for *Chara/Potamogeton* (fig. 5).

DISCUSSION

Freshwater benthic communities are influenced by a wide spectrum of biotic and abiotic factors (Round 1971). Benthic diatom distribution in lakes is driven by microhabitat

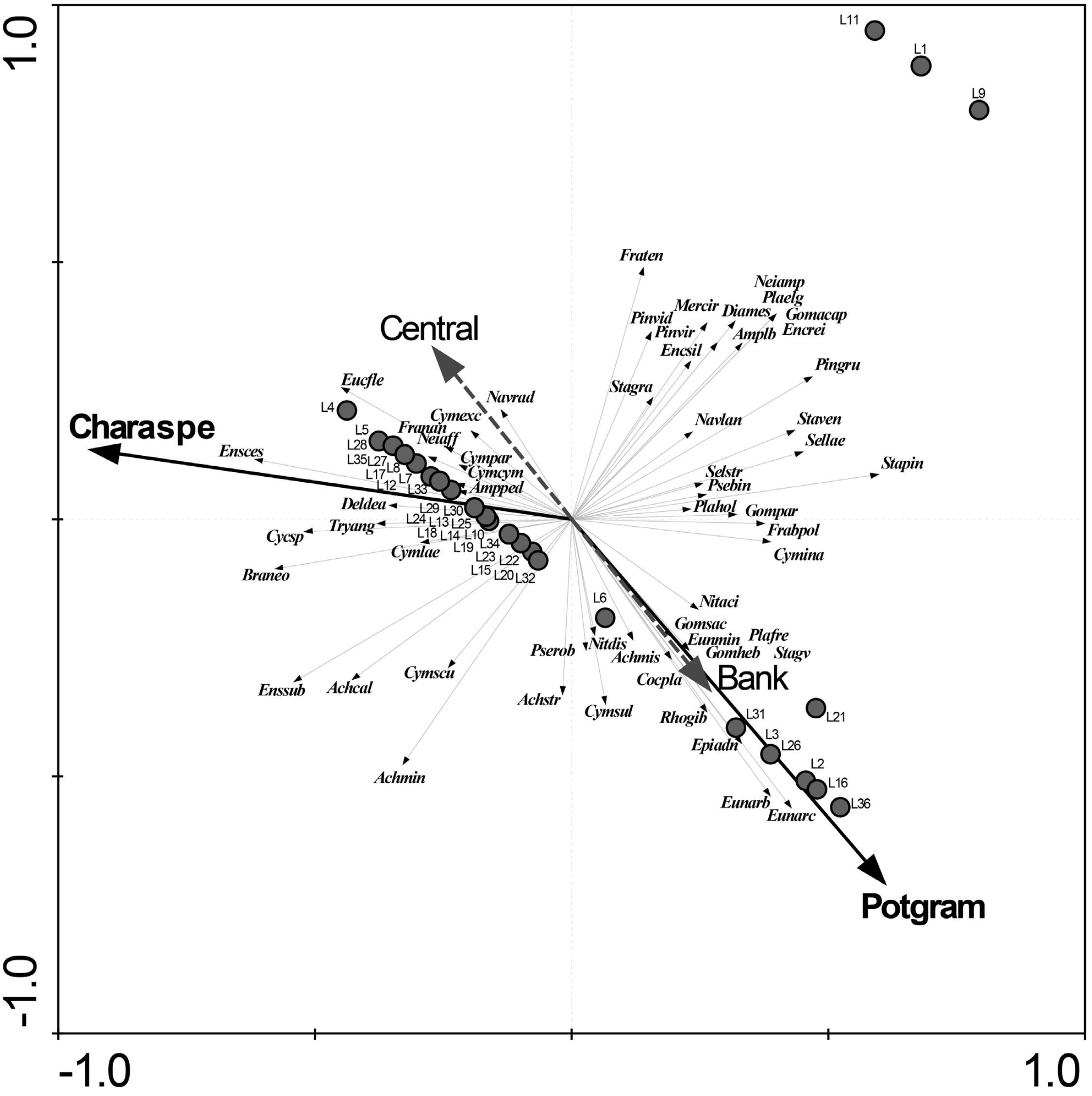

Figure 3 – Redundancy Analysis: L1–L36 sampling sites (see table 3; abbreviations of species see electronic appendix), Charaspe – *Chara aspera*, Potgram – *Potamogeton gramineus*, Central - central part of the lake), Bank - first sample of each transect situated at the lake bank: L1, L5, L6, L10, L11, L15, L16, L20, L21, L25, L26, L30, L31, L36). F = 7.024, p = 0.004.

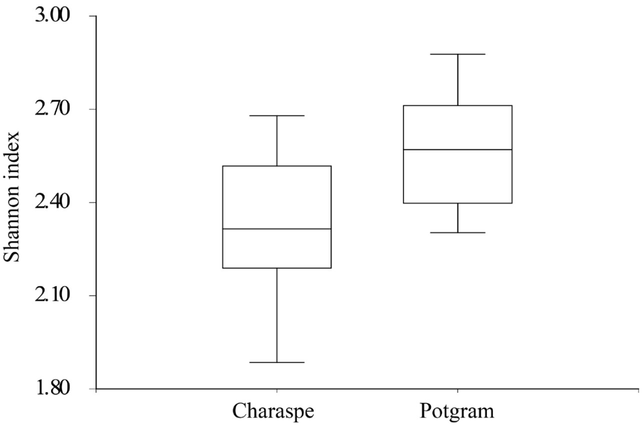

Figure 4 – One way ANOVA: epiphytic diatom diversity (Shannon index) on *Chara aspera* (Charaspe) and *Potamogeton gramineus* (Potgram). F = 9.01, p = 0.0053.

distribution, and depends upon hydrological situation (Neif et al. 2013), lake bathymetry, light (Cano et al. 2012), nutrient and substrate availability (Cantonati et al. 2009, Cano et al. 2012) and grazing (Meerhoff et al. 2007). Some results show that epiphytic diatom communities respond mainly to physical/chemical variables, and only secondarily to lake depth, size, and location (Blanco et al. 2014). Plankton in the limnetic zone have primary access to solar light, whereas benthic associations in the littoral zone to nutrients released by mineralization processes in the sediment (Wetzel 1996). The decrease of light intensity with water depth is certainly influenced by dispersed particles in the water column, and high phytoplankton abundance dramatically decreases the depth distribution of benthic algae by shading. Although competition for light between plankton and microphytobenthos has been discussed in some Alpine lakes (Poulíčková et al. 2004, Cantonati et al. 2009), phytoplankton seems to be a poor competitor in Lake Valagola (table 2 cf. chlorophyll *a* concentration).

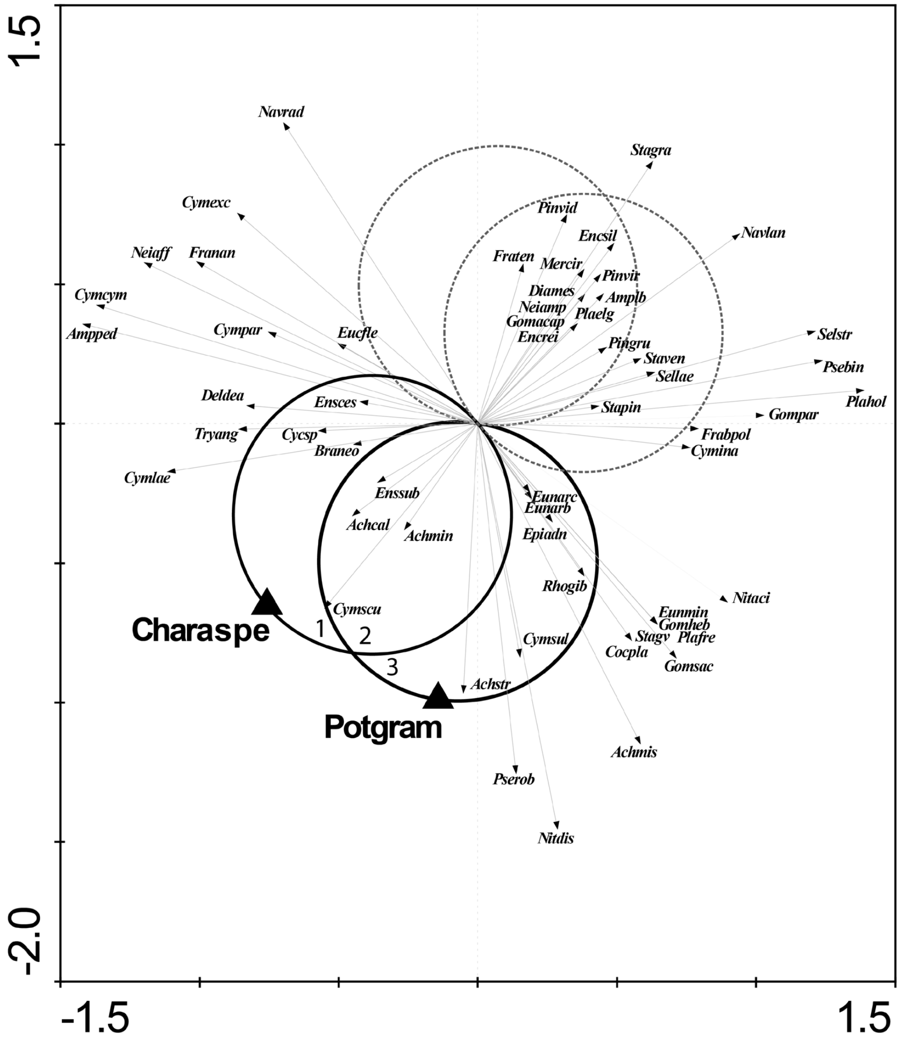

Figure 5 – T-value biplot diagram showing species sorted into Van Dobben circles showing positive or negative influence of substrate (Charaspe - *Chara aspera*, Potgram - *Potamogeton gramineus*). See electronic appendix for species names and abbreviations. Species inside circles responded significantly to the substrate. Black circles indicate positive responses, and gray circles indicate negative responses. Van Dobben circles in the case of *Chara and Potamogeton* partially cover each other, which means that in zone 2 are species colonizing both substates. Zone 1 represents species colonizing *Chara aspera*, zone 3 represents species colonizing *Potamogeton gramineus*.

Dramatic changes in epiphyton biomass and structure observed along transects across deep lakes from the littoral to the limnetic zone (Yang et al. 2009) were not expected in our shallow, clear lake. However, the differences between central and marginal parts are still detectable, even though a true limnetic zone is missing (fig. 3). Indeed, similar differences between the central and peripheral zone were found in other European lakes/ponds and have been explained by macrophyte density, animal and wind disturbances, wave action or solar radiation (Cano et al. 2012, Kitner et al. 2005, Poulíčková et al. 2006) irrespective to their depth.

In contrast to substrate specificity, depth influence was not found to be significant in this study (analysis not illustrated). However, some differences found in this study can be explained by the biology and ecology of both dominant macrophytes. Growth and development of *Potamogeton* follows an annual cycle (with regrowth in spring that starts from tubers or from buds on relic stems; e.g. Wiegleb & Kadono 1989) whilst *Chara* is a perennial with apical growth (e.g. Krause 1997).

The western shore of Lake Valagola is dominated by *Potamogeton* (depth 0.5 to 2.2 m) whereas *Chara aspera* grows in the rest of the lake. Their spatial distribution should be explained by competition for light and free CO_2, as previously reported for *C. aspera* and *Potamogeton pectinatus* L. (van den Berg et al. 1998). However, *Stuckenia pectinata* (L.) Börner (previously *Potamogeton pectinatus*) and *P. gramineus* differ significantly and van den Berg's model consider much more turbid conditions. The depth distribution of macrophytes in Lake Valagola suggests that *C. aspera* is tolerant to shading. This is in agreement with records highlighting that charophytes colonize deeper parts than angiosperms (Blindow 1992). Although depth distribution cannot explain why *Potamogeton* colonizes only the western shore of the lake, light could again be the possible factor, because the eastern shore of the tarn is shaded by wood, while the western shore is more open.

Both dominant macrophytes (*Chara aspera* and *Potamogeton gramineus*) host significantly different diatom species assemblages. Diatom species richness, diversity, and composition differed significantly between these two main host plants. Moreover, the diversity hot spot (L21; *Calliergon giganteum*; fig. 1B) might be influenced by groundwater inflow (Cantonati et al. 2012). *Potamogeton gramineus* assemblages were characterized by higher species richness and diversity, and by the large-celled, adnate diatom species *Epithemia adnata*, *Rhopalodia gibba*, *Eunotia arcus*, and *E. arcubus*. *Chara aspera* was preferred by the small-celled, motile diatom species *Brachysira neoexilis*, *Encyonopsis cesatii*. As we documented by measurements and host plant surface SEM images, *Chara* thalli seems to be a more difficult surface for diatom collonization, particularly for longer species (fig. 6).

Because macrophyte distribution is spatially structured in this lake, we can hardly separate differences induced by substrate/host from other possible influences. Substrate preferences caused either by morphology of plant species (mechanical cause; Laugaste & Reunanen 2005, Pomazkina et al. 2012) or nutrient uptake from host plants (chemical cause) are accompanied by seasonal aspects of epiphyte distribution.

Seasonal changes have been found to be significant in shallow lowland ponds (Kitner et al. 2005). Final cover and rate of colonization was higher in summer than in spring.

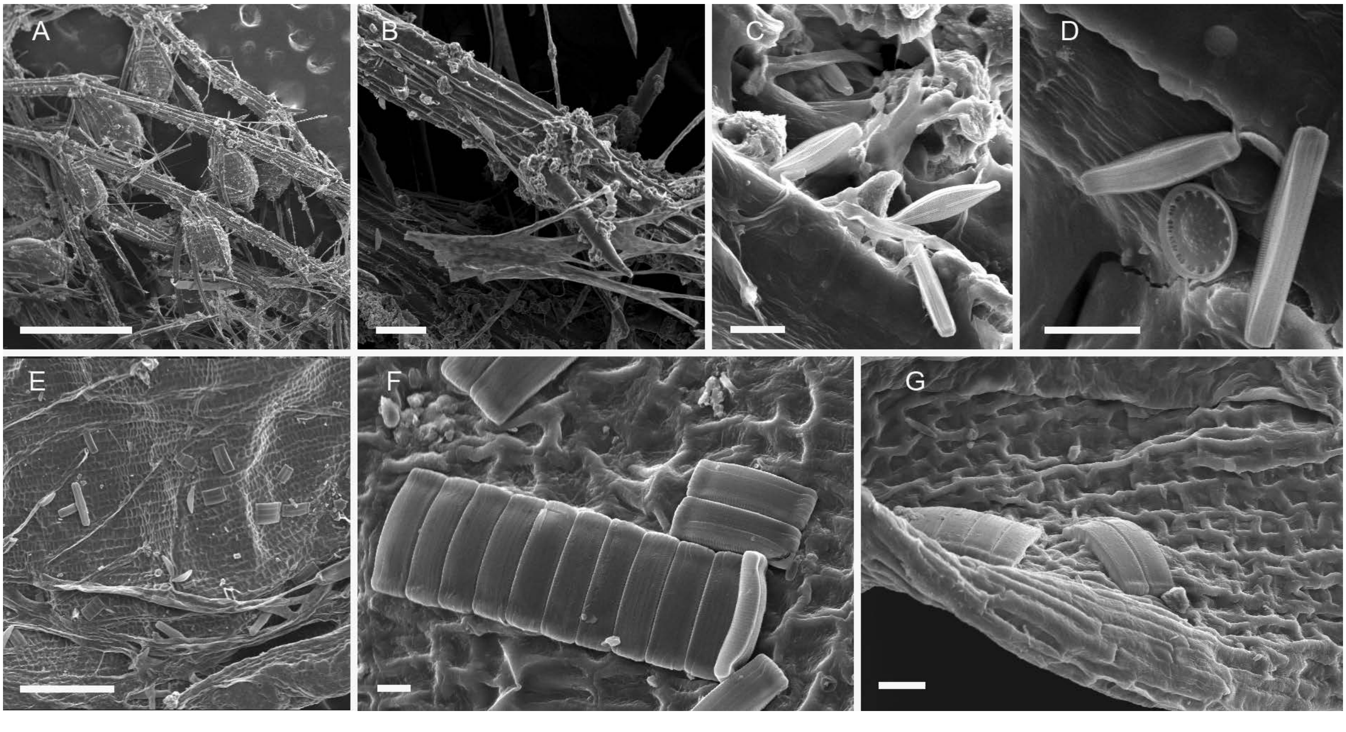

Figure 6 – SEM images of herbarium material of *Chara aspera* (A–D) and *Potamogeton gramineus* (E–G) showing diatom epiphytes on the surface. Scale bars: A, 1 mm; B, 100 µm; C, D & F, 10 µm; E, 200 µm; G, 20 µm.

Differences have been explained by temperature and light fluctuations (Hoagland et al. 1982, Kitner et al. 2005). Moreover, seasonal changes influence the growth of macrophytes in terms of surface for algal colonization (Pizarro 1999). Seasonal differences caused by hydrological dynamics have been verified in shallow floodplain lakes (Neif et al. 2013).

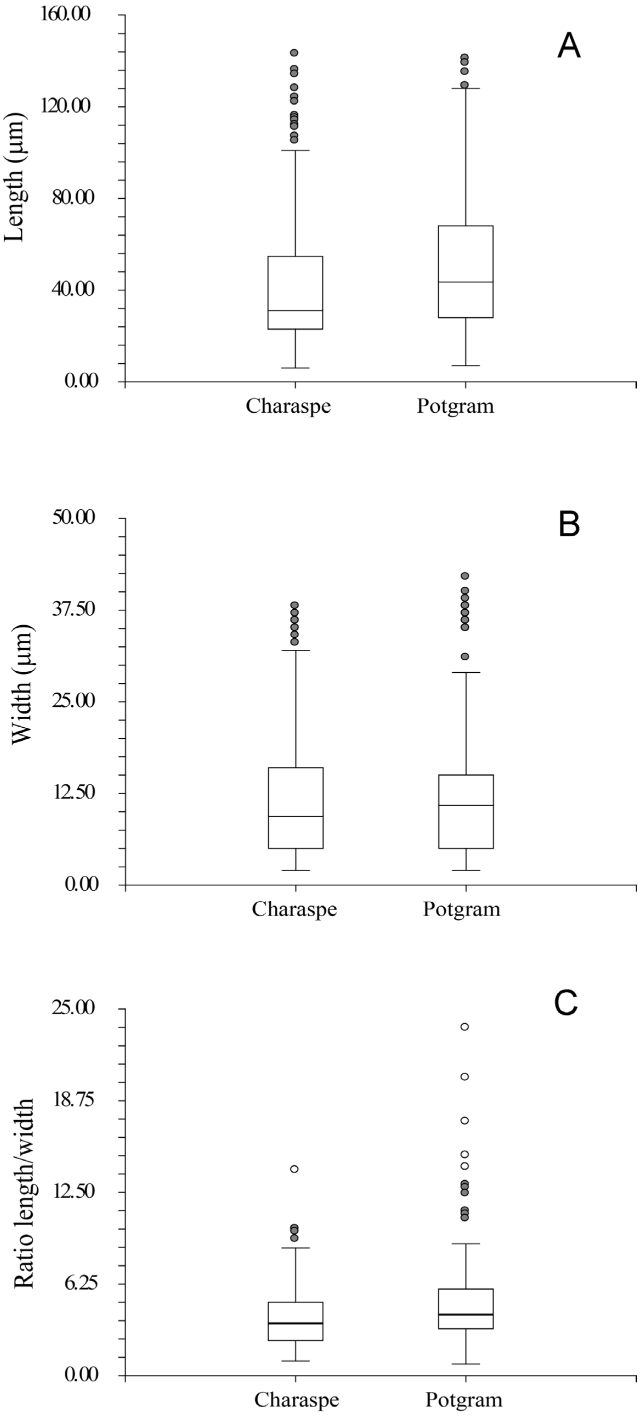

Figure 7 – One way ANOVA: variability of epiphytic diatom size structure found on *Chara aspera* (Charaspe) and *Potamogeton gramineus* (Potgram). A, diatom cell length (F = 11.49, p = 0.0008); B, diatom cell width (F = 1.64, p = 0.2010); C, length/width ratio (F = 12.39, p = 0.0005).

The vegetative season in Lake Valagola is shorter (about 6 months) because of the elevation, and the lake was sampled at the end of the summer when colonization of substrata and assemblage development are maximal in temperate mountain lakes (Catalan & Donato Rondón 2016). The architecture of the host plant is undoubtedly of significance, particularly, in lakes with low nutrient levels. According to Hinojosa-Garro et al. (2010) macrophyte architectural complexity leads to an increase of the epiphytic species richness and diversity. In highly-eutrophic lakes, substrate specificity can be less pronounced (Eminson & Moss 1980, Kairesalo 1984, Kitner & Poulíčková 2003, Laugaste & Reunanen 2005). No qualitative or quantitative specificity for substrata was observed in eutrophic ponds and streams in Czech Republic (Kollár et al. 2015) or in a study on Lake Erie (Millie & Lowe 1983).

However, Cejudo-Figueiras et al. (2010) rejected Blindow's (1987) neutral substrate hypothesis, and observed significant differences in the composition of diatom assemblages among host macrophytes. In contrast, diatom-based indices for trophic level assessment did not differ significantly. Thus, they hypothesize that epiphytic diatoms can be used as indicators for shallow lakes irrespective of the dominant macrophyte (Cejudo-Figueiras et al. 2010).

As we expected, we found a significantly higher diversity on *Potamogeton*, more likely due to appropriate plant architecture and surface, which is in agreement with the opinion of other authors (Pomazkina et al. 2012). The development of epiphyton can be affected also by allelopathic interactions (Gross 2003). Whereas cyanobacteria are strongly inhibited by compounds produced by *Chara aspera*, surprisingly no inhibition was noted in eukaryotic target strains including one diatom strain (Berger & Schagerl 2003, 2004).

In contrast to some studies mentioned above, our results support the existence of substrate specificity for diatom assemblages. Although, such distinct model cases as *Lemna* spp. vs. *Lemnicola hungarica* (Grunow) Round & Basson seem to be rare (Buczkó 2007). *Chara aspera* and other Charophyceae of the Balkan Peninsula were inhabited by other diatom species (Hafner & Jasprica 2013), because the localities were brackish. Variation in species composition of epiphyton growing on *Potamogeton* and other macrophytes (*Myriophyllum* sp., *Elodea* sp.) were rarely studied (Pomazkina et al. 2012). The most common epiphytic diatom seems to be *Cocconeis placentula* Ehrenberg (Birkett & Gardiner 2005, Potapova & Charles 2005, Lebreton et al. 2009). We found it to be more common on *Potamogeton* rather than on *Chara*. *Cocconeis* preferring shores exposed to wind with active wave mixing (Kozhov 1962, Pomazkina et al. 2012) was accompanied in Lake Valagola by diverse species of *Epithemia* and *Rhopalodia*.

In conclusion, our work pointed out general features of diatom assemblages colonizing two macrophytes with contrasting depth-preferences and architectures, particularly in terms of size structure of epiphytic diatoms.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, supplementary data site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data) and consist of a list of species with abbreviations and host plants.

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4.3 Paper IV

KOLLÁR J., FRÁNKOVÁ M., HAŠLER P., LETÁKOVÁ M. & POULÍČKOVÁ A. (2015): Epiphytic diatoms in lotic and lentic waters – Diversity and representation of species complexes. *Fottea* 15 (2): 259 – 271.

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Epiphytic diatoms in lotic and lentic waters – diversity and representation of species complexes

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Abstract: Small streams and shallow ponds represent sensitive ecosystems and attached diatoms can serve as integrative indicator with fast response to environmental changes. Development of methods for ecological monitoring throughout Europe and their calibration for particular ecoregions are not finished yet and databases need to be filled by data from undersampled regions and overlooked substrates. The present study aims to explore the diversity of epiphytic diatoms in unexplored catchment areas with special attention to substrate specificity and distribution of unresolved diatom species complexes. Significant differences were found in diversity of both regions and water types (lotic/lentic). No significant differences were found in the case of substrates. CCA analysis showed significant influence of pH, water streaming (streaming/stagnant) and *Lemna* substrate to species composition. Surprisingly species complexes represent the majority of epiphytic assemblages with no significant differences between lotic and lentic waters or substrates except of *Lemna*. The high representation of complexes does not lead automatically to reduction of overall diversity of the sample.

Key words: diatoms, epiphyton, lotic and lentic waters, species complexes

Introduction

In Europe, most shallow lakes/ponds and rivers are strongly affected by human activities. The EU members in the frame of Water Framework Directive (WFD) developed standardized methods to assess the ecological status of surface waters using bioindicators. Diatoms are considered to be good indicator organisms in aquatic ecosystems (Blanco et al. 2004, 2014). The cross–taxon congruence of six contrasting groups of organisms (vascular plants, bryophytes, fungi, diatoms, desmids and testate amoebae) in the same permanent plots were analysed in freshwater wetlands (Hájek et al. 2014). The main difference among different taxa corresponded clearly with body size and life span (micro versus macroorganisms), conforming the assumption of faster response of microorganisms to environmental changes. Generally, macroorganisms provide similar information, while diatoms behave most independently (Hájek et al. 2014). Diatoms occupy a variety of substrates in both lotic and lentic waters. Development of methods for ecological monitoring throughout Europe (Kelly et al. 2009) and their calibration for particular ecoregions are not finished yet and databases need to be filled by data from undersampled regions and less sampled substrates. Moreover there are many problems with cryptic diversity and their ecological significance (Poulíčková et al. 2008, 2014). Some diatom traditional morphospecies included in regional floras (*Sellaphora pupula*, *Achnanthidium minutissimum*, *Gomhonema parvulum* etc.) have long been considered cosmopolitan, ubiquitous, and morphologically highly variable taxa. However molecular methods revealed, that these diatoms are species complexes consisting of few or many species, whose identification in LM is difficult or impossible (Potapova & Hamilton 2007; Mann et al. 2008; Poulíčková et al. 2010). The use of benthic diatoms in the context of ecological status assessment (King et al. 2006; Kelly et al. 2007) seems to be broadly accepted, although more studies are dealing with running waters and epilithon (Rimet & Bouchez 2012). Methodology for shallow lakes using epiphyton has been suggested quite recently (Blanco et al. 2014). The present study aims to explore epiphytic diatoms of small ponds and streams covering main ecological gradients of Southeastern Moravia (Czech Republic). Special attention was paid to representation of diatom species complexes at different substrates and water types and its influence to epiphyton bioindication capacity.

Material and Methods

Samples were collected in summer 2013 and 2014 in 25 ponds and 13 streams of two sampling areas. Both regions (the Svitava region and the White Carpathian Mountains) belong to the Morava River Basin. The first one – the Svitava Highland is a part of the Svitava River basin (SB) and geologically belongs to the southeastern part of the Cretaceous Table. In the area prevail mesozoic (sandstone, marstone, marlstone, claystone) and quarternary (loam, loess, gravel, sand) sedimentary rocks. Annual mean temperature is around 6 °C and annual mean precipitaion is around 600 mm (Tolasz et al. 2007). Sites located in this area lay in elevation around 500 m a.s.l. (Fig. 1). The second area – the White Carpathian Mountains (WC) is situated on southeast of the Czech Republic (on the western margin of the Western Carpathians) along the border with Slovakia. Geological bedrock is formed by flysch belt, in which sandstone and claystone of variable calcium content alternate. Prevailing is marl, lime–rich claystone, limestone and calcareous sandstone (Hájek et al. 2002). Groundwaters are carbonatogenic and their dominant mineralization process is carbonate dissolution which leads to the calcium–(magnesium)–bicarbonate type of chemistry (Rapant et al. 1996). This chemistry type supports cold water travertine (tufa) formation. Annual mean temperature is about 8°C and annual mean precipitaion is about 700 mm (Tolasz et al. 2007). Sites located in this area are situated in altitudes from 225 m up to 535 m above sea level. Basic characteristics of investigated localities are given in Table 1.

Epiphytic communities (in littoral part of ponds and/or streaming part of the rivers) growing on submerged macrophytes *Phragmites australis* (Cav.) Steud.; *Poaceae* (incl. *Phalaris arundinacea* L., *Arrhenatherum elatius* (L.) J. Presl et C. Presl, *Poa* sp., *Dactylis* sp., *Glyceria* sp.); *Typha* sp.; *Lemna* sp.; *Salix* sp.; *Callitriche* sp. and *Sparganium* sp. were examined.

Diatom sampling methods followed those reccommended by Kelly et al. (1998), diatom frustules were cleaned in hydrogen peroxide (Taylor et al. 2007) and mounted in Naphrax. Four hundred individuals were identified in each sample to species level using literature (Krammer & Lange–Bertalot 1986; Krammer & Lange–Bertalot 1988; Krammer & Lange–Bertalot 1991; Krammer 2000; Lange–Bertalot 2001; Krammer 2002; Krammer 2003; Krammer & Lange–Bertalot 2004; Lange–Bertalot et al. 2011). Nomenclature has been unified following Algaebase (Guiry & Guiry 2015). Species complexes selection was based on actual list of species, recent molecular literature and own experience and are summarized in Table 2, although their list can expand in near future due to molecular studies boom. Few examples of species complexes representatives are documented in Fig. 2.

Statistical analysis. Our hypothesis assumes that diatom distribution among sampling sites is influenced by measured environmental variables. Prior to main statistical analyses we disproved correlation between geographical position and environmental variables (Spearman´s correlation coefficient, pH: $r_s = 0.587$, conductivity: $r_s = 0.033$). Multivariate analysis using Canoco 4.5 (ter Braak & Šmilauer 2002) was carried out to test relationships between identified diatoms and environmental variables (pH, conductivity, host plant, streaming/stagnant water). First, *length* of the gradient was computed using Detrended correspondence analysis (DCA, detrending by segmnents, without transformation, length of the gradient on the first axis = 5.258, speceies data explain 11.8% on the first axis). Protocol of the Canonical correspondence analysis (CCA) was carried out as follows: imported data included diatom occurrence (%) and environmental variables (pH, conductivity, flowing/stagnant water, host plant), then biplot scaling focused on inter–species distances, Log transformation (Y´=log(A*Y+B), A=1, B=1) with downweighting of rare species, Monte–Carlo permutation test was used (reduced model, 499 permutations), forward seletion of environmental variables (both automatic and manual selection) were performed. Analysed environmental variables did not show any collinearity. Their VIF ranged from 1.82 to 5.92. Results of CCA were visualized using Canoco Draw

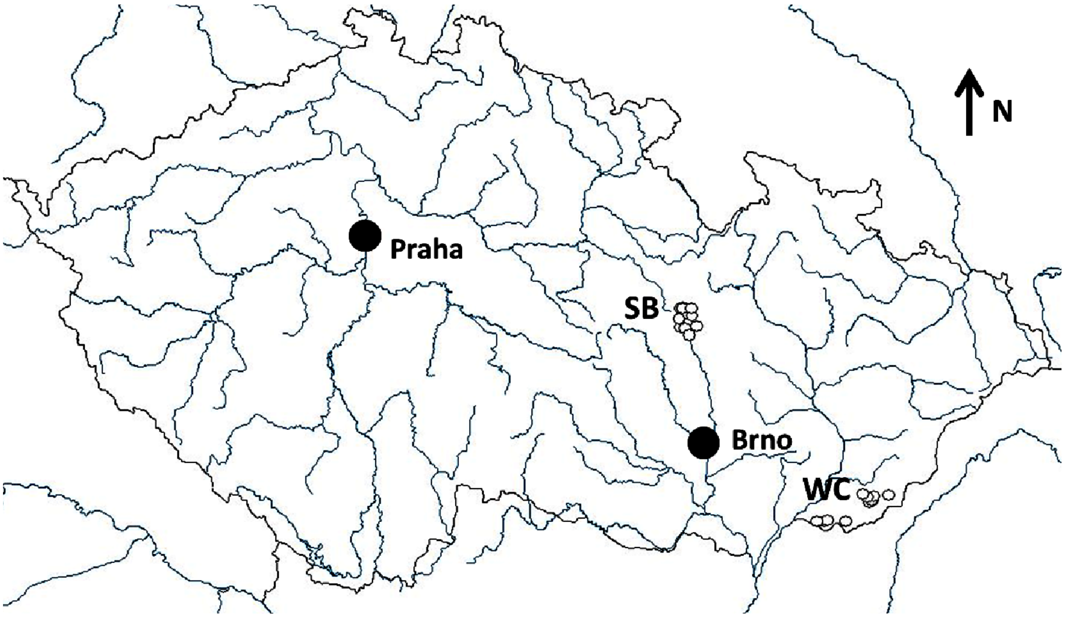


Fig. 1. Sampling sites in White Carpathians (WC) and the Svitava river basin (SB).

Table. 1. Basic characteristics of investigated localities, (Cond) conductivity (μmS.cm^{-1}).

Area	Sample	Locality (Cadaster)	GPS coordinates	pH	Cond	Pond/ stream	Macrophyte
WC	LOK1	Lučina (Tvarožná Lhota)	48°51'46.14"N 17°23'41.04"E	8.19	505	P	*Poaceae*
WC	LOK2	Kejda (Kněždub)	48°52'3.84"N 17°24’37.26”E	8.13	470	P	*Poaceae*
WC	LOK3	Radějovka (Radějov)	48°51'38.16"N 17°20'32.70"E	8.32	546	S	*Poaceae*
WC	LOK4	Hrubý potok (Javorník)	48°51'49.56"N 17°31'54.24"E	8.36	414	S	*Poaceae*
WC	LOK5	Rasová (Komňa)	48°58‘32.40“N 17°48’43.86”E	8.24	317	P	*Typha*
WC	LOK6	Lubná (Suchá Loz)	48°56'47.88"N 17°40'54.60"E	7.93	451	P	*Sparganium*
WC	LOK7	Lubná (Suchá Loz)	48°56'32.16"N 17°40'55.80"E	8.09	523	S	*Poaceae*
WC	LOK8	Basin on the Hradecký járek (Suchá Loz)	48°57'1.68"N 17°42'14.58"E	7.79	368	P	*Typha*
WC	LOK9	Hradecký járek (Suchá Loz)	48°57'8.52"N 17°42'5.88"E	7.92	408	S	*Salix*
WC	LOK10	Nivnička (Suchá Loz)	48°58'13.20"N 17°42'36.30"E	8.06	539	S	*Poaceae*
WC	LOK11	Basin near Čupák (Suchá Loz)	48°57'53.52"N 17°40'23.34"E	8.13	430	P	*Typha*
WC	LOK12	Nivnička (Nivnice)	48°58'48.00"N 17°38'30.84"E	8.45	547	S	*Poaceae*
SB	Ra1	Radiměřský potok (Radiměř)	49°41'31.163"N 16°27'26.189"E	6.2	175	S	*Poaceae*
SB	HnS1	Dolní hradecký rybníček (Hradec nad Svitavou)	49°41'7.646"N 16°28'57.071"E	7.85	743	P	*Phragmites*
SB	HnS2	Horní hradecký rybníček (Hradec nad Sitavou)	49°41'8.094"N 16°28'55.294"E	4.80	534	P	*Lemna*
SB	HnS4	Řeka Svitava (Hradec nad Svitavou)	49°41'6.744"N 16°28'51.934"E	7.00	540	S	*Poaceae*
SB	Sy1	Lánský rybník (Svitavy – Lány)	49°44'35.760"N 16°28'8.220"E	6.3	385	P	*Phragmites*
SB	Sy2	Svitavský rybník (Svitavy – Lačnov)	49°45'58.719"N 16°27'37.682"E	6.60	401	P	*Phragmites*
SB	Sy3	Rosnička (Svitavy – Předměstí)	49°46'15.313"N 16°27'5.582"E	7.98	506	P	*Phragmites*
SB	Sy6	Svitavy (Svitavy – Lány)	49°44'36.775"N 16°28'41.705"E	6.90	528	S	*Phragmites*
SB	Sy8	Lačnovský západní rybník (Svitavy – Lačnov)	49°46'25.950"N 16°28'7.276"E	5.75	293	P	*Typha*
SB	Sy12	Outlet at Lačnovský západní rybník (Svitavy – Lačnov)	49°46'24.584"N 16°28'10.762"E	6.00	251	P	*Typha*

Table 1 Cont.

SB	V1	U Rybníčku (Vendolí)	49°43'33.575"N 16°26'39.028"E	6.30	148	P	*Lemna*
SB	Po2	Fishpond (Pohledy)	49°41'46.008"N 16°33'39.107"E	6.30	252	P	*Phragmites*
SB	KH1	Fishpond (Kamenná horka)	49°44'18.342"N 16°31'43.116"E	5.80	459	P	*Typha*
SB	K1	Pool (Koclířov)	49°46'20.363"N 16°31'21.760"E	5.88	332	P	*Phragmites*
SB	BnS1	Svitava (Březová nad Svitavou)	49°39'25.761"N 16°30'27.857"E	6.10	552	S	*Poaceae*
SB	Br1	Svitava (Brněnec)	49°37'23.628"N 16°31'26.769"E	7.65	518	S	*Phragmites*

Table 2. Species complexes occurring in the White Carpathians and the Svitava Basin, their trophic preferences, (nd) no data available.

Species complex	**References**	**Trophic state (Van Damm et al. 1994)**
Achnanthidium minutissimum	Potapova & Hamilton 2007	euryvalent
Planothidium lanceolatum	Van de Vijver et al. 2013	eutrophic
Amphora pediculus	Bruder 2006, Wang 2014	eutrophic
Cocconeis pediculus	Jahn et al. 2007	eutrophic
Cocconeis placentula	Jahn et al. 2009	eutrophic
Encyonema minutum		nd
Eunotia bilunaris	Vanormelinger et al. 2013	euryvalent
Ulnaria ulna	Williams 2011	euryvalent
Fragilaria capucina	Kahlert et al. 2009	euryvalent
Staurosirella pinnata	Morales et al. 2013	euryvalent
Gomphonema parvulum	Abarca et al. 2014	eutrophic
Navicula cryptocephala	Poulíčková et al. 2010	euryvalent
Nitzschia palea	Kahlert et al. 2009; Trobajo et al. 2009	hypertrophic
Nitzschia paleacea		eutrophic
Sellaphora pupula	Mann et al. 2004, 2008; Vanormelingen et al. 2013	mesotrophic

4.0. Shannon diversity indexes of diatoms were computed (then sorted according to: host plants, sampling site, streaming/stagnant water, geographical location of sampling sites). Variation of Shannon diversity indexes among sampling sites and streaming/stagnant water was analysed with One–Way ANOVA. Because of unequal number of host plants sampled, variation of Shannon diversity index was analysed with non– parametric Kruskal–Wallis multiple comparison test (NCSS statistical package, Hintze 2007). With respect to different plant habitus and physiology, difference in Shannon´s diversity was analysed for *Lemna minor* versus group of other submerged vascular plants. Response of diatoms to the best fitting environmental variables was anlysed using T–value statistics (CanocoDraw 4.0).

RESULTS

A total of 131 diatom species was found during the study. Species richness ranged from 1 to 34 species per sample. Species richness was higher in the West Carpathians (19–34) than in the Svitava river basin (1–15 per sample). The highest number of diatom species was found on *Poaceae* and *Typha*. The dominant diatom species was *Achnanthidium minutissimum* agg. creating up to 88% of the community. The most frequently occuring species were *Gomphonema parvulum* agg. with representation 1–48% and *Cocconeis placentula* with representation 1–100%. Frequent species for both regions were also *Nitzschia palea* and *Ulnaria ulna*. Planktonic diatoms (*Cyclotella, Aulacoseira* and *Asterionella*) frequently occurred in ponds.

Surprisingly species complexes (Table 2) represent the majority of epiphytic assemblages (lotic/lentic: 67,1% and 66,5% respectively; substrates: *Poaceae* 63%, *Typha* 60%, *Sparganium* 79,2%, *Salix* 74.9%, *Phragmites* 75%, *Callitriche* 89,8%, *Lemna* 25%). However, differences in percentage of species complexes among sampled host plants were not significant (F = 1.91, P = 0.1341). There was no close correlation between Shannon´s diversity and percentage of species complexes among sampled host plants as well (Pearson correlation coefficient: r = 0.4113) by other words: the high representation of complexes does not automatically lead to reduction of overall diversity of the sample.

Canonical correspondence analysis spread sampling points through the ordination spacewith respect to their geographical position (West Carpathians and Svitava river basin) and ecological nature (stream and pond). Sampling sites in the Western Carpathians, both ponds and streams, form more coherent clusters than in the Svitava river basin (Fig 3). Significant differences in Shannon´s diversity index were found between sampling sites in the Svitava river basin and Western Carpathians (Fig. 6, F = 5.88, p = 0.0204). Sampling sites located in the Western Carpathians (Fig. 3, squares) possess statistically higher Shannon diversity (1.95±0.55) than those in the Svitava river basin (1.43±0.68). Similarly, statistically significant differences were found between sampling sites from streaming (2.08±0.47) and stagnant water bodies (1.33±0.63; Fig. 5, F = 13.94, p = 0.0006).

Species data explain 11.8% of variability on the first and 19.8% on the second ordination axis (p = 0.002, F = 3.792). Diatom ordination is significantly influenced by pH (F = 3.84, p = 0.002), water hydrodynamism (streaming/stagnant, F = 2.59, p = 0.002) and *Lemna minor* as a host plant (F = 1.97, p = 0.018, for details see Table 3, Fig. 4). Species such as *Amphora pediculus*, *Cocconeis pediculus*, *Cymbella excisiformis*, *Encyonopsis cesatii*, *Encyonopsis microcephala*, *Eunotia arcus*, *Gomphonema pumilum*, *Nitzchia palaeformis* or *Nitzchia sinuata* prefer significantly higher pH than *Mayamaea atomus*, *Planothidium ellipticum*, *Planothidium lanceolatum* or *Nitzchia palea*, which prefer lower pH value within investigated scale (4.80–8.45). Species preferring stagnant water bodies include *Fragilaria brevistriata*, *Encyonopsis microcephala*, *Eunotia arcus* and *Denticula tenuis*. On the other hand, streaming water prefer *Cocconeis pediculus*, *Gomphonema angustatum* and *Navicula tripunctata*. Diatom assemblages among sampled host plants possess almost the same diversity. *Lemna minor* showed the lowest variability of Shannon diversity (1.37±0.17) in contrast to other host plants (Phrag = 1.56±0.72, Poac = 1.82±0.53, Salix = 1.54±1.38, Typha = 1.50±0.59). However, Shannon´s diversity did not show any statistically significant differences among host plants (Table 4). Similarly, difference in Shannon´s diversity between *Lemna minor* and other vascular submerged plants was not significant as well (z = 0.874, α = 0.05). Diatoms showed low specificity to host plants except of *Lemna minor* (F = 1.97, p = 0.018). Species such as *Fragilaria brevistriata*, *Staurosirella pinnata* or *Nitzschia palaeformis* avoid *Lemna minor* as a host plant. Surprisingly, *Lemnicola hungarica* as a diatom typical for *Lemna minor*, inhabited also *Phragmites australis* in the Svitava river basin.

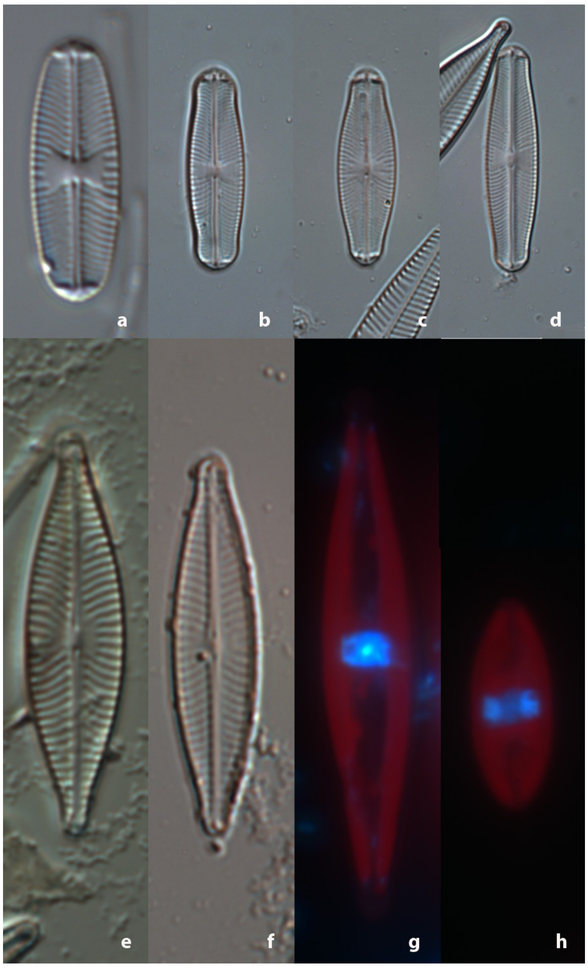


Fig. 2. Examples of species complexes in the Czech Republic: (a–d) *Sellaphora pupula* sensu lato differ in frustule morphology, (e–h) *Navicula cryptocephala* sensu lato differ in interphase nuclei structure.

Discussion

Small streams and shallow ponds represent ecosystems sensitive to environmental changes. It can be demonstrated by much higher nutrient variation in shallow than deep waters (Jeppesen et al. 2000). In comparison with physicochemical variables, attached diatoms seem to be more integrative indicators with fast response to environmental changes (Blanco et al. 2004; Hájek et al. 2014). Diatoms are able to inhabit all available substrates, which are mostly represented by

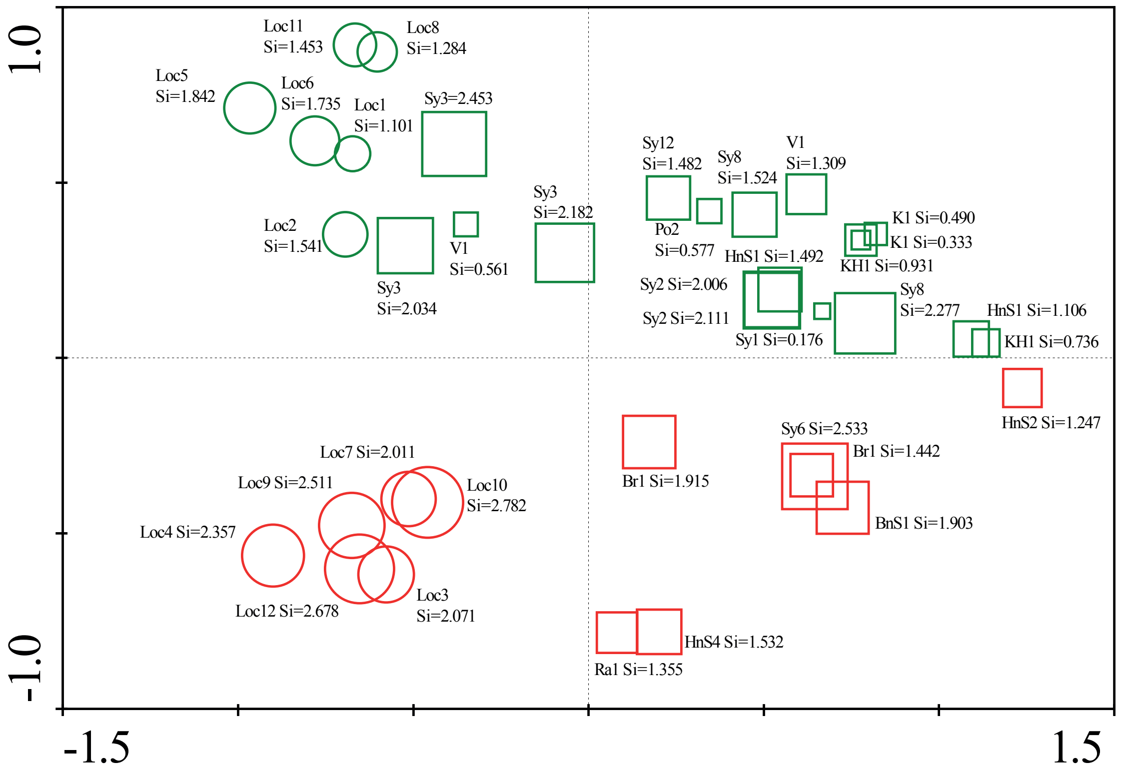
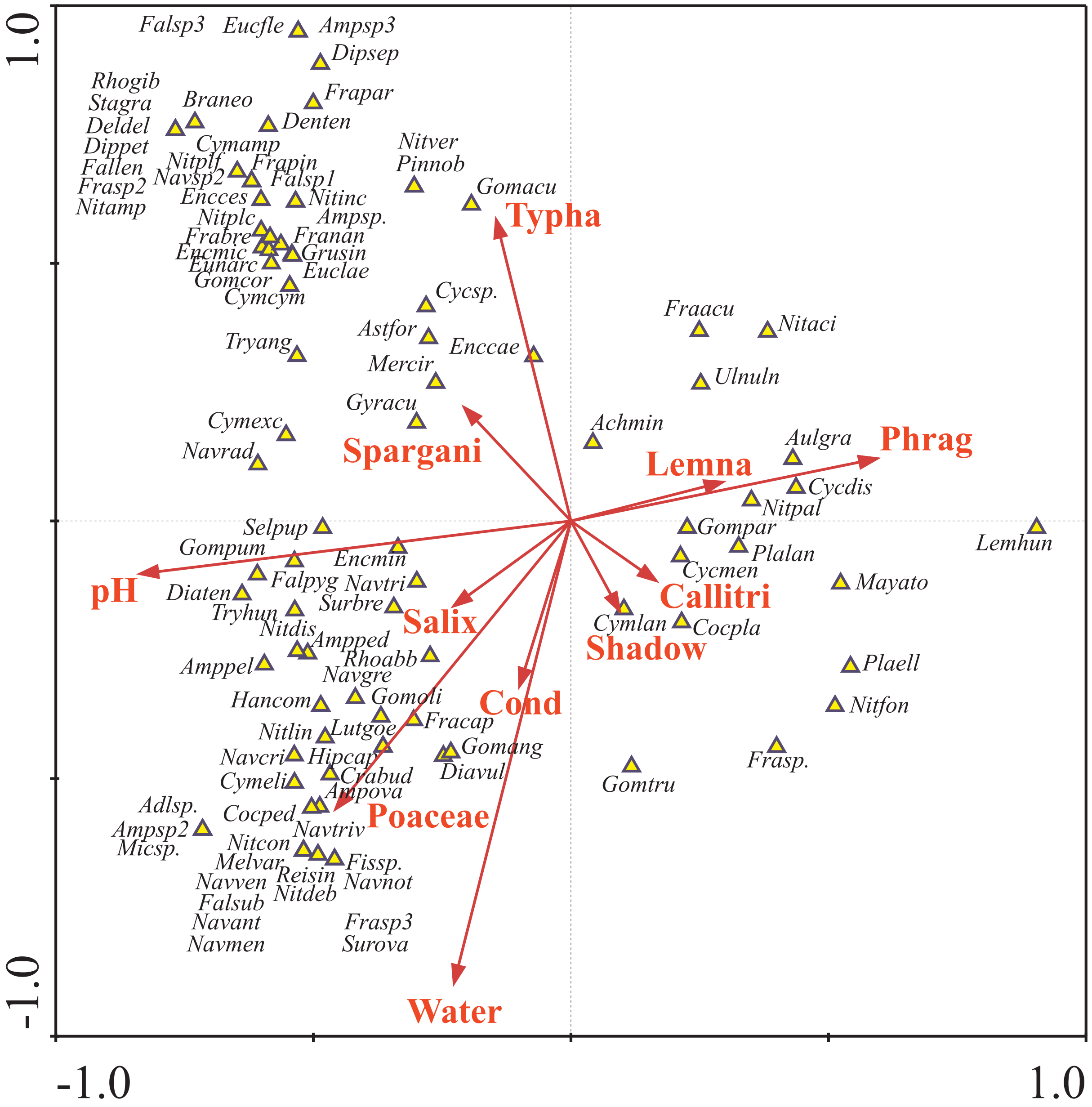


Fig. 3 CCA ordination diagram: investigated sites, (circles) Western Carpathians, (squares) Svitava river basin, (green) ponds, (red) streams; symbol size corresponds to value of Shannon´s diversity index (Si).

Fig. 4. CCA ordination biplot diagram: species vs. environmental variables: (Achmin) *Achnanthidium minutissimum* (Kützing) Czarnecki, (Adlsp) *Adlafia* sp, (Ampel) *Amphipleura pellucida* (Kützing) Kützing, (Ampova) *Amphora ovalis* (Kützing) Kützing, (Ampped) *Amphora pediculus* (Kützing) Grunow ex A. Schmidt, (Ampsp) *Amphora sp,* (Ampsp2) *Amphora sp. 2*, (Ampsp3) *Amphora* sp. *3*, (Astfor) *Asterionella formosa* Hassall, (Aulgra) *Aulacoseira granulata* (Ehrenberg) Simonsen, (Braneo) *Brachysira neoexilis* Lange–Bertalot, (Cocped) *Cocconeis pediculus* Ehrenberg, (Cocpla) *Cocconeis placentula* Ehrenberg, (Cabud) *Craticula buderi* (Hustedt) Lange–Bertalot, (Cyccom) *Handmannia comta* (Ehrenberg) Kociolek et Khursevich, (Ccdis) *Cyclotella distinguenda* Hustedt, (Cycmen) *Cyclotella meneghiniana* Kützing, (Cycsp) *Cyclotella* sp., (Cymeli) *Cymatopleura elliptica* (Brébisson) W. Smith, (Cymcym) *Cymbella cymbiformis* C. Agardh, (Cymexc) *Cymbella excisiformis* Krammer, (Cymlan) *Cymbella lanceolata* (C.Agardh) Kirchner, (Cymamp) *Cymbopleura amphicephala* (Nägeli) Krammer, (Deldel) *Delicata delicatula* (Kützing) Krammer, (Denten) *Denticula tenuis* Kützing, (Diaten) *Diatoma tenuis* agg., (Diavul) *Diatoma vulgaris* Bory de Saint–Vincent, (Dippet) *Diploneis petersenii* Hustedt, (Dipsep) *Diploneis separanda* Lange–Bertalot, (Enccae) *Encyonema caespitosum* Kützing, (Encmin) *Encyonema minutum* agg., (Encces) *Encyonopsis cesatii* (Rabenhorst) Krammer, (Encmic) *Encyonopsis microcephala* agg., (Eucfle) *Eucocconeis flexella* (Kützing) Meister, (Euclae) *Eucocconeis laevis* (Østrup) Lange–Bertalot, (Eunarc) *Eunotia arcus* Ehrenberg, (Fallen) *Fallacia lenzii* (Hustedt) Lange–Bertalot, (Falpyg) *Fallacia pygmaea* (Kützing) A.J. Stickle et D.G. Mann, (Falsp1) *Fallacia* sp., (Falsp3) *Fallacia* sp. *3*, (Falsub) *Fallacia subhamulata* (Grunow) D.G. Mann, (Fissp) *Fistulifera* sp., (Fraacu) *Fragilaria acus* (Kützing) Lange–Bertalot, (Frabe) *Fragilaria brevistriata* Grunow, (Fracap) *Fragilaria capucina* Desmazières, (Franan) *Fragilaria nanana* Lange–Bertalot, (Frapar) *Fragilaria parasitica* (W. Smith) Grunow var. *parasitica*, (Frasp) *Fragilaria* sp., (Frapin) *Staurosirella pinnata* (Ehrenberg) D. M. Williams et Round, (Frasp2) *Fragilaria* sp. *2*, (Frasp3) *Fragilaria sp*., (Gomacu) *Gomphonema acuminatum* Ehrenberg, (Gomang) *Gomphonema angustatum* (Kützing) Rabenhorst, (Gomcor) *Gomphonema coronatum* (Ehrenberg), (Gomoli) *Gomphonema olivaceum* (Hornemann) Brébisson, (Gompar) *Gomphonema parvulum* (Kützing) Kützing, (Gompum) *Gomphonema pumilum* (Grunow) E. Reichardt et Lange–Bertalot, (Gomtru) *Gomphonema truncatum* Ehrenberg, (Gyracu) *Gyrosigma acuminatum* (Kützing) Rabenhorst, (Hipcap) *Hippodonta capitata* (Ehrenberg) Lange–Bertalot, Metzeltin et Witkowski, (Lemhun) *Lemnicola hungarica* (Grunow) F.E.Round et P.W.Basson, (Lutgoe) *Luticola goeppertiana* (Bleisch) D.G. Mann, (Mayato) *Mayamaea atomus* (Kützing) Lange–Bertalot, (Melvar) *Melosira varians* C. Agardh, (Mercir) *Meridion circulare* (Greville) C.Agardh, (Micsp) *Microcostatus* sp., (Navant) *Navicula antonii* Lange–Bertalot, (Navci) *Navicula cryptotenelloides* Lange–Bertalot, (Navgre) *Navicula gregaria* Donkin, (Navmen) *Navicula menisculus* Schumann, (Navnot) *Navicula notha* Wallace, (Navrad) *Navicula radiosa* Kützing, (Navsp2) *Navicula* sp. *2*, (Navtri) *Navicula tripunctata* (O.F. Müller) Bory de Saint–Vincent, (Navtriv) *Navicula trivialis* Lange–Bertalot, (Navven) *Navicula veneta* Kützing, (Nitaci) *Nitzschia acicularis* (Kützing) W.Smith, (Nitamp) *Nitzschia amphibia* Grunow, (Nitang) *Tryblionella angustata* W. Smith, (Nitcon) *Nitzschia constricta* (Kützing) Ralfs, (Nitdis) *Nitzschia dissipata* (Kützing) Rabenhorst, (Nitfon) *Nitzschia fonticola* (Grunow) Grunow, (Nithun) *Tryblionella hungarica* (Grunow) Frenguelli, (Nitfru) *Nitzschia inconspicua* Grunow, (Nitlin) *Nitzschia linearis* W. Smith, (Nitplf) *Nitzschia palaeformis* Hustedt, (Nitdeb) *Nitzschia palea* var. *debilis* (Kützing) Grunow, (Nitpal) *Nitzschia palea* (Kützing) W.Smith, (Nitplc) *Nitzschia paleacea* Grunow, (Nitsin) *Grunowia sinuata* (Thwaites) Rabenhorst, (Nitver) *Nitzschia vermicularis* (Kützing) Hantzsch, (Pinnob) *Pinnularia nobilis* (Ehrenberg) Ehrenberg, (Plaell) *Planothidium ellipticum* (Cleve) Round et Bukhtiyarova, (Plalan) *Planothidium lanceolatum* (Brébisson ex Kützing) Bukhtiyarova, (Reisin) *Reimeria sinuata* (Gregory) Kociolek et Stoermer, (Rhoabb) *Rhoicosphenia abbreviata* (C.Agardh) Lange–Bertalot, (Rhogib) *Rhopalodia gibba* (Ehrenberg) Otto Müller, (Selpup) *Sellaphora pupula* (Kützing) Mereschkowsky, (Stagra) *Stauroneis gracilis* Ehrenberg, (Surbre) *Surirella brebissonii* var. *kuetzingii* Krammer et Lange–Bertalot, (Surova) *Surirella ovalis* Brébisson, (Ulnuln) *Ulnaria ulna* (Nitzsch) P.Compère. Variables: (Water) water streaming 0/1, (Cond) conductivity, pH, (Shadow) percentage of shade 0%/50%/100%; substrates: *Phragmites*, Poaceae, *Typha, Lemna, Salix, Callitriche, Sparganium*.



stones and sediments in streams and by macrophytes and sediments in shallow lakes and ponds. Homogeneity of periphytic communities and their composition are more related to chemical characteristics of the surrounding environment than to the substrate type, particularly in eutrophic systems (Eminson & Moss 1980; Cattaneo et al. 1998; Kitner & Poulíčková 2003; Poulíčková et al. 2004; Cejudo–Figueiras et al. 2010). However, substrate specificity has been described in some oligotrophic waters (Eminson & Moss 1980; Blindow 1987; Buczkó 2006; Cantonati 1998; Poulíčková et al. 2004). Our results did not confirm substrate specificity, except of specific assemblage growing on *Lemna* sp., similar to more complex study already published (Buczkó 2007). In contrast to other periphytic assemblages, epiphytic assemblages include lower number of planktonic diatom taxa and suspended particules (Kelly et al. 1998, Poulíčková et al. 2004, 2008, 2014). Planktonic diatoms were not frequent and were represented by *Aulacoseira granulata* (SB), *Asterionella formosa* and *Cyclotella* sp. (WC). In general, the relationship between epiphyton and water chemistry has been demonstrated many times (Ács et al. 1991, 1994; Kitner & Poulíčková 2003; Blanco et al. 2004; Poulíčková et al. 2004; Hájková et al. 2011) and submerged macrophytes have been recommended for routine monitoring (Kelly et al. 1998; Blanco et al. 2004). We found significant relationship to selected environmental variables (water streaming, pH) in congruence with other studies (Potapova & Charles 2003; Kitner & Poulíčková 2003; Kovács et al. 2006; Fránková et al. 2009; Yang & Flower 2012).

However, the results of this method of water quality status assessment are strongly influenced by following

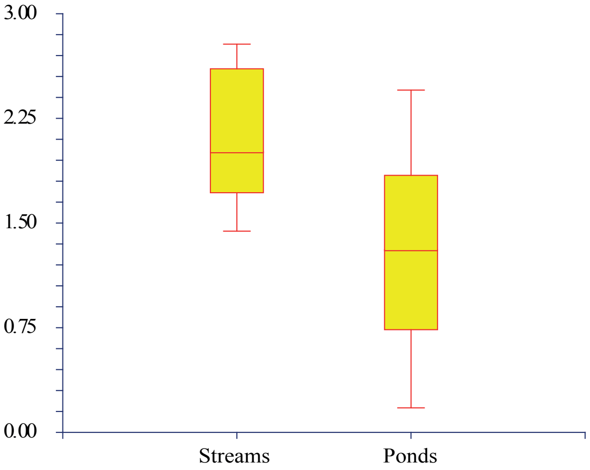


Fig. 5. Box plot of diatom Shannon diversity: comparison of habitat (streams, ponds; F=13.94, p=0.0006).

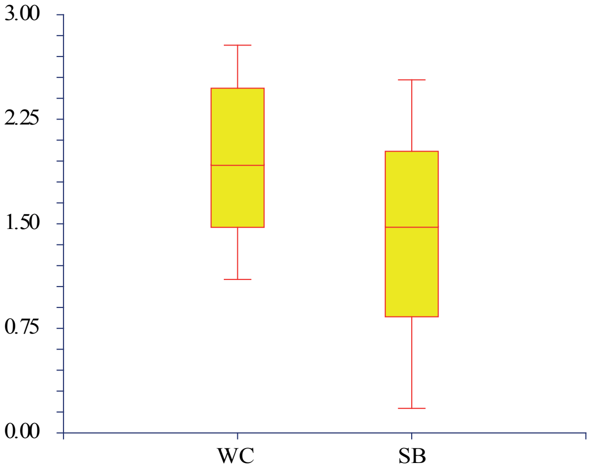


Fig. 6. Box plot of diatom Shannon diversity: comparison of sampling sites (SB) Svitava river basin, (WC) Western Carpathians (F=5.88, p=0.0204).

two factors: 1) trophic indices are working in ecoregions where they were intercalibrated (Prygiel et al. 2002; Poulíčková et al. 2004; Kovác et al. 2006) and 2) some traditional „euryvalent and cosmopolitan species“ represent species complexes consisting of few or many ecologically differentiated biological species (so called cryptic species), whose distinguishing in the LM is difficult or even impossible (Mann et al. 2008; Kahlert et al. 2009; Poulíčková et al. 2010).

Although many studies noticed that attached diatoms in wide spectrum of ecological conditions are dominated particularly by *Achnanthidium minutissimum* (Kützing) Czarnecki 1994 (Blanco et al. 2004, Cejudo–Figueiras et al. 2010), the assessment what is the proportion of species complexes within attached diatom assemblages has not been specified yet. Sigee (2005) summarized dominant diatom species along a river course with increasing nutrient pollution. First zone (clean water) is characterized by small–celled species directly attached to stone surface (*Eunotia exigua, Achnanthes microcephala*). Zones 2 and 3 are dominated by *Fragilaria capucina*, *Achnanthidium minutissimum*, *Encyonema minutum* and *Cocconeis placentula*. Eutrophic zone 4 is characterized by *Gomphonema parvulum* and highly eutrophic zone 5 by *Nitzschia palea* (Sigee 2005). In fact the majority of these „indicatory dominants“ represent species complexes (Table 2; Kwandrans et al. 1998; Kahlert et al. 2009). If species complexes as a whole are not ecologically differentiated and create the majority of assemblage composition, it means that in this field is a great potential for trophic indices improvement. The ongoing progress with identification of cryptic diversity is in motion with implementation of molecular methods. Following six examples demonstrates the importance of species complexes investigation.

Sellaphora pupula agg.

An extreme example of species complexes seems to be *Sellaphora pupula* agg. (Fig. 2; Mann et al. 2008) with almost 50 morphospecies (demes), some of them already confirmed using molecular methods (Evans et al. 2007, 2009; Wetzel et al. 2015). This diatom is typical for epipelic rather than epiphytic assemblages, with high representation in British lakes. It creates up to 40% of epipelic assemblages in lakes/ponds of Great Britain, while its representation in Czech and Hungarian ponds does not exceed 3% (Poulíčková et al. 2008, Špačková et al. 2009). Although their identification is difficult particularly in the LM, some of them seem to be ecologically differentiated (Poulíčková et al. 2008). Many lakes contain several different morphospecies, the greatest numbers of coexisting demes occurred in eutrophic Blackford Pond, Great Britain (Poulíčková et al. 2008). Five morphospecies inhabiting Czech pond Bezedník (temperate zone) showed seasonal dynamics with significant correlation of their occurrence with temperature (Špačková et al. 2009).

Achnanthidium minutissimum agg.

Although molecular methods have not been used in this case yet, the opinion that previously described varieties within *A. minutissimum* can represent ecologically differenctiated species seems to be evident (Potapova & Hamilton 2007). Moreover this species complex has been recorded as the most frequent dominant of epilithic and epiphytic assemblages in both lotic and lentic freshwaters (Ponader & Potapova 2007; Potapova & Hamilton 2007). Morphometric study (Potapova & Hamilton 2007) revealed 6 morphological groups, however authors were not able to draw clear boundaries between them. These morphospecies differred significantly in their ecology and could serve as indicators of water quality (Potapova & Hamilton 2007). However, an analysis of the results of 25 diatomists participating in intercalibration exercise showed, that even experienced diatomists have problem to recog-

Table 3. CCA forward selection of environmental variables: influence of environmental variables on species distribution in the Svitava river basin and the White Carpathian Mts [(Water) streaming/stagnant water, (Cond) conductivity, (Lemna) *Lemna minor*, (Spargani) *Sparganium* sp., (Shadow) shadow/half–shadow/light, (Typha) *Typha* spp., (Phrag) *Phragmites australis*, (Callitri) *Callitriche* sp.].

Conditional Effects					**Marginal Effects**		
Variable	**Var.N**	**LambdaA**	**p**	**F**	**Lambda1**	**p**	**F**
pH	1	0.32	**0.002**	**3.84****	0.32	**0.002**	**3.84****
Water	4	0.21	**0.002**	**2.59****	0.22	**0.004**	**2.50****
Cond	2	0.16	**0.010**	**2.10****	0.13	0.090	1.41
Lemna	8	0.15	**0.018**	**1.97****	0.17	**0.024**	**1.88****
Spargani	11	0.10	0.254	1.27	0.11	0.358	1.21
Shadow	3	0.08	0.278	1.14	0.09	0.406	1.1
Typha	7	0.08	0.314	1.7	0.13	0.078	1.51
Poaceae	6	0.08	0.434	1.1	0.18	**0.004**	**2.3****
Salix	9	0.08	0.452	1.00	0.09	0.464	0.99
Phrag	5	0.03	0.920	0.41	0.20	**0.002**	**2.33****
Callitri	10				0.05	0.796	0.57

** statistically significant

Table 4. Kruskal–Wallis Multiple–Comparison Z–Value Test (Dunn's Test), differences of Shannon´s diversity index among host plants [(Lemna) *Lemna minor*, (Poac) *Poaceae*, (Phrag) *Phragmites australis*, (Salix) *Salix* spp., (Typha) *Typha* spp.].

	Lemna	**Poac**	**Phrag**	**Salix**	**Typha**
Lemna	0.0000	1.1867	0.8036	0.5970	0.4306
Poac	1.1867	0.0000	0.6897	0.4315	1.1457
Phrag	0.8036	0.6897	0.0000	0.0269	0.5636
Salix	0.5970	0.4315	0.0269	0.0000	0.3141
Typha	0.4306	1.1457	0.5636	0.3141	0.0000

Regular Test: Medians significantly different if z–value > 1.9600
Bonferroni Test: Medians significantly different if z–value > 2.8070

nize varieties/morphospecies of *A. minutissimum* in the LM (Kahlert et al. 2009), due to small size close to the LM resolution limits (length 5 – 25 μm, width 2.5 – 4 μ and dense striation 26–30/10μm; Hofmann et al. 2013). Improvement in bioassessments in this case strongly depends on application of molecular methods.

Gomphonema parvulum agg.

The name *G. parvulum* represents a diatom species which is relatively small in size (length 10–36μm, width 5–8μm) and has cosmopolitan distribution (Hofmann et al. 2013). In fact it has been used as a collective name for a species complex for two centuries. Morphologically highly variable diatom occurrs in wide range of water qualities (Patrick & Reimer 1975; Hudstedt 1985; Krammer & Lange–Bertalot 1997). Molecular as well as morphological data obtained during the recent studies (Kermarec et al. 2013; Abarca et al. 2014) resulted in separation of at least four taxa based on their biogeography.

Eunotia bilunaris agg.

E. bilunaris sensu lato is a good candidate for studies on semicryptic species diversity in diatoms. It is a cosmopolitan and common epiphytic diatom in oligotrophic, mainly acidic freshwater bodies (Krammer & Lange–Bertalot 1991; Vanormelingen et al. 2008). Based on its phenotypic plasticity, a number of species have been described (Lange–Bertalot et al. 2011). Moreover morphological, molecular and reproductive data suggest the existence of several reproductivelly isolated species (Varnormelingen et al. 2008).

Navicula cryptocephala agg.

N. cryptocephala is a common benthic diatom of moderate size (20–40 um long, 5–7 μm wide; Lange–Bertalot 2001).

In contrast to species complexes with broad morphological variation, *N. cryptocephala* represents a complex with almost identical valve morphology. However, it has been found to be polymorphic with respect to interphase nuclear structure (Fig. 2; Geitler 1951, 1952a,b, 1958; Poulíčková et al. 2010). Phylogenetic analyses of 52 strains confirmed the existence of two genetically distinct lineages within *N. cryptocephala* that coexist sympatrically and are widely distributed, occurring in European and Australian ponds (Poulíčková et al. 2010).

Nitzschia palea **agg.**

N. palea is believed to be a widely distributed diatom in lotic and lentic freshwater habitats (Finlay et al. 2002; Potapova & Charles 2007). In general, the genus *Nitzschia* is difficult for identification and discrimination between members, particularly in the section Lanceolatae Grunow (Hustedt 1930). Moreover identification is complicated by morphological variability during the life cycle and phenotypic plasticity due to environmental conditions. On the base of their results (morphological, genetic and mating diversity) Trobajo et al. 2009 concluded that *N. palea* is not a simple, homogeneous taxon and that this complex will probably have to be split into three or more species. At least two of them appear to be geographically widespread. Ecological preferences and potential indicatory value need to be further investigated (Trobajo et al. 2009).

In conclusion, species complexes are important, because of their common occurrence, frequent dominance and difficulties with their distinguishing.

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4.3 Paper V

POULÍČKOVÁ A., LETÁKOVÁ M., HAŠLER P., COX E. & DUCHOSLAV M. (2017): Species complexes within epiphytic diatoms and their relevance for the bioindication of trophic status. *Science of the Total Environment* 599-600: 820-833.

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Species complexes within epiphytic diatoms and their relevance for the bioindication of trophic status

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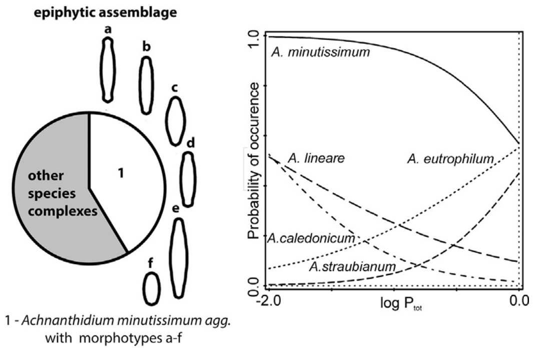
HIGHLIGHTS

- Freshwater diatoms were examined at localities along a trophic gradient.
- Number of indicator species increased with fine taxonomic resolution.
- Species complexes showed low sensitivity to changes in phosphorus concentration.
- Cryptic species had contrasting relationships to trophic gradient.
- Some cryptic species have potential to improve bioassessment models.

GRAPHICAL ABSTRACT



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ABSTRACT

The popularity of aquatic bioassessments has increased in Europe and worldwide, with a considerable number of methods being based on benthic diatoms. Recent evidence from molecular data and mating experiments has shown that some traditional diatom morphospecies represent species complexes, containing several to many cryptic species. This case study is based on epiphytic diatom and environmental data from shallow fishponds, investigating whether the recognition and use of fine taxonomic resolution (cryptic species) can improve assessment of community response to environmental drivers and increase sharpness of classification, compared to coarse taxonomic resolution (genus level and species level with unresolved species complexes). Secondly, trophy bioindication based on a species matrix divided into two compartments (species complexes and remaining species) was evaluated against the expectation that species complexes would be poor trophy indicators, due to their expected wide ecological amplitude. Finally, the response of species complexes and their members (cryptic species) to a trophic gradient (phosphorus) were compared.

Multivariate analyses showed similar efficiency of all three taxonomic resolutions in depicting community patterns and their environmental correlates, suggesting that even genus level resolution is sufficient for routine bioassessment of shallow fishponds with a wide trophic range. However, after controlling for coarse taxonomic matrices, fine taxonomic resolution (with resolved cryptic species) still showed sufficient variance related to the environmental variable (habitat groups), and increased the sharpness of classification, number of indicator species for habitat categories, and gave better separation of habitat categories in the ordination space. Regression

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Abbreviations: P_{tot}, total phosphorus concentrations; SPC, species complexes; RSP, remaining species; GL, genus level resolution; SLC, species level resolution with unresolved species complexes; SL, species level resolution with cryptic species recognized; DCA, detrended correspondence analysis; CCA, canonical correspondence analysis; CoCA, co-correspondence analysis; pCCA, partial canonical correspondence analysis; ANOVA, analysis of variance.

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analysis of trophic bioindication and phosphorus concentration showed a weak relationship for species complexes but a close relationship for the remaining taxa. GLM models also showed that no species complex responded to phosphorus concentration. It follows that the studied species complexes have wide tolerances to, and no apparent optima for, phosphorus concentrations. In contrast, various responses (linear, unimodal, or no response) of cryptic species within species complexes were found to total phosphorus concentration. In some cases, fine taxonomic resolution to species level including cryptic species has the potential to improve data interpretation and extrapolation, supporting recent views of species surrogacy.

1. Introduction

Cryptic species can be defined as two or more distinct species that are classified as a single taxon (Bickford et al., 2007), a “species complex” or “aggregate”. Cryptic species are morphologically similar, superficially indistinguishable, but separable with molecular data or other techniques (Poulíčková et al., 2016). Cryptic species have been recognized for nearly 300 years but research into them has increased exponentially over the past three decades due to the increasing availability of DNA sequences (Bickford et al., 2007). Cryptic diversity is recorded from various groups of organisms (Bickford et al., 2007; Fernandez et al., 2006; Funk et al., 2016; Trontelj and Fišer, 2009), including microalgae and diatoms (Degerlund et al., 2012; Kaczmarska et al., 2014; Kulichová and Fialová, 2016).

Bickford et al. (2007) surveyed the literature for references containing the phrases “cryptic species” or “sibling species” in the title, abstract or keywords, and found surprisingly few papers reporting cryptic species in higher plants or microbes. Botanists do not use these phrases, but species complexes due to polyploidy in angiosperms are common (e.g., Dančák et al., 2012; Duchoslav et al., 2013; Husband et al., 2013; Kobrlová et al., 2016; Soltis et al., 2007). Barker et al. (2015) recently estimated that there are ca 50–60,000 cryptic polyploid species in angiosperms that await discovery and naming. Microorganismal molecular data are still limited because of problems associated with DNA isolation. To obtain sufficient material microorganisms must be grown up in clonal culture. Single cell/filament PCR potentially solves this problem but is time consuming. In addition, some microorganismal groups have cell wall structures that complicate DNA isolation, e.g. different types of frustules, loricas, and particularly different types of mucilage envelope (Mareš et al., 2015; Mazalová et al., 2011).

Many reasons have been given for the value of recognizing cryptic species, e.g. biodiversity, conservation, disease treatment and bioprospecting (Barker et al., 2015; Geller, 1999). Bioindication and biomonitoring are based on organisms having known, distinctive ecological requirements, or specific responses to environmental stressors, such as pollution and nutrient enrichment (Adams, 2002; Diekmann, 2003; Zonneveld, 1983). Aquatic bioassessments have increased in popularity in Europe, methods being based mostly on macrophytes and benthic invertebrates (54%), phytoplankton (21%), fish (15%) and phytobenthos (10%; Birk et al., 2012). Bioindication requires standardized sampling, sample processing and identification of collected organisms, mostly (74%) to species level (Birk et al., 2012). However, species complexes are often common, widespread and euryvalent (Poulíčková et al., 2008b; Schlick-Steiner et al., 2007) and thus less suitable (Diekmann, 2003). The importance of recognizing cryptic species is relevant when entities within species complexes are ecologically differentiated (Poulíčková et al., 2008b). Knowledge of the bioindication value of cryptic species could improve the sensitivity of bioassessment methods, particularly for the European Water Framework Directive (European Commmission, 2000), which requires an assessment of ecological quality at an ecoregional level (Rimet and Bouchez, 2012a).

Diatoms are widespread organisms playing a key role in all freshwater ecosystems (Round, 1981). Therefore, they are considered powerful indicators for recent and past water quality and climates (Birks et al., 1990; Smol and Stoermer, 2010). Recent evidence from molecular data and mating experiments has shown that some traditional diatom morphospecies represent species complexes containing several to many cryptic species (Kulichová and Fialová, 2016). *Sellaphora pupula* agg., a characteristic epipelic taxon (Supplementary Fig. S1), is an excellent example of diatom cryptic diversity, with >50 morphotypes (probably cryptic species) in Great Britain alone (Mann et al., 2008), some of which have been found to be ecologically differentiated with respect to trophic gradients (Poulíčková et al., 2008b). *Achnanthidium minutissimum* agg. (Supplementary Fig. S1) is another example of cryptic diversity. Although many ecological studies have reported its dominance (lentic vs lotic freshwaters, epiphyton, epilithon; Rimet and Bouchez, 2012a; Cantonati et al., 2014), few have tried to document cryptic diversity within this complex (Potapova and Hamilton, 2007; Wojtal et al., 2011). Together with a few other complexes, *A. minutissimum* can dominate stream and pond epiphyton, contributing up to 97% of the assemblage (Kollár et al., 2015; Supplementary Table S1), not a trivial percentage.

Ignoring taxonomic heterogeneity and potential ecological differentiation within such species complexes may bias ecological assessments of water quality, despite evidence that evaluations adopting lower taxonomic resolution (genus, family, life-forms or guilds; ‘taxonomic sufficiency’ or ‘taxonomic surrogates’; Terlizzi et al., 2003) show similar, or even better pollution assessment than species-level resolution (e.g., Growns, 1999; Hill et al., 2001; Rimet and Bouchez, 2012b). Indeed, a requirement for cost-effective methods for elucidating the response of ecosystems (across terrestrial, freshwater and marine environments) to anthropogenic impacts has focused on the use of higher taxa as surrogates for species (reviewed by Terlizzi et al., 2003), stemming from the idea of phylogenetic niche conservatism (Losos, 2008; Webb et al., 2002; Keck et al., 2016). However, our knowledge of the extent to which ecological similarity is related to phylogenetic relatedness among species is generally limited (Bevilacqua et al., 2012), partly because the taxonomic classification of many organisms does not mirror phylogenetic relatedness (Wheeler, 2004). More recently, Bevilacqua et al. (2012, 2013) found that the statistical power to detect environmental changes with coarse taxonomic resolution depends on the degree of species aggregation (i.e., the higher taxa/species ratio) rather than the taxonomic resolution (see also Siqueira et al., 2012). Due to the discovery of closely related taxa (cryptic species) (Bickford et al., 2007) that can exhibit ecological niche differentiation (Vanelslander et al., 2009), the continual increase in the number of diatom species may decrease the usefulness of coarse taxonomic resolution for the assessment of community responses to environmental variables. In this respect, Bevilacqua et al. (2012, 2013) suggested combining species surrogacy (difficult taxa, tolerant species) with the retention of high taxonomic detail when necessary (indicator taxa, sensitive species, easy recognizable species). Following these ideas we focused on the potential utility of diatom cryptic species for improving the identification of community response to environmental variation. The philosophy of this study is based on expectation that species complexes are generally euryvalent and blur community response to environmental variation, while cryptic species should be ecologically differentiated and thus might be usefull for the detection of (subtle) community responses to environment. In our case study based on epiphytic diatom and environmental data from shallow fishponds we tested the following null hypotheses:

1. Three levels of taxonomic resolution, genus (GL), species level with unresolved complexes (SLC), species level with cryptic species (SL), are equally effective in revealing community response to environmental variation and do not show significant differences in assessing the main environmental gradients.
2. After splitting the species matrix into species complexes and remaining species, there is no difference in the trophy bioindication of both parts of the epiphyton assemblages.
3. Species complexes and their members (cryptic species) share the same ecological responses to trophy.
4. Using finer taxonomic resulution did not increase sharpness of classification, i.e. number of indicator taxa for habitat categories with contrasting trophic levels relative to taxonomic richness.

2. Material and methods

2.1. Localities, sampling and measurements

Fifty-two samples were taken from 40 ponds in the Czech Republic during September 2013, and May, June and July 2014. Epiphytic diatoms were sampled from one of the following substrata: macrophytes *Typha angustifolia* L., *Epilobium hirsutum* Huds., *Carex* sp., *Salix* sp., *Phragmites australis* Trin. ex Steud., *Lemna minor* Griff., *Carex vesicaria* Leers, *Lysimachia thyrsiflora* Geners, *Juncus effusus* Auct. Am. ex Schult. F., *Polygonum amphibium* L., Poaceae; and microphytes *Oedogonium* sp., *Cladophora* sp. If more then one substratum occurred at a sampling site, more samples were taken, from each of the respective substrata (for sample and locality details see Supplementary Table S2, Hašler et al. 2008, Poulíčková et al. 2008a).

Diatom samples with their substratum were placed in plastic bags. In the laboratory all the contents, including the substratum, were transferred into Erlenmayer flasks with 30% H_2O_2 and left for 24 h to start the oxidation process slowly (as described in Letáková et al., 2016). The samples with hydrogen peroxide were then boiled until the volume decreased by two thirds, and a few milligrams of $K_2Cr_2O_7$ and 1 ml of concentrated HCl were added into the hot liquid. All the chemicals were washed out through careful rinsing with distilled water until the final pH was neutral. Clean diatom samples were mounted in Naphrax (two slides per sample). Diatoms were investigated by light microscopy using a Zeiss 'Primo star' (Germany), firstly qualitatively and then semiquantitatively (relative abundance as %). At least 400 diatom valves were counted per sample according to European standards (Kelly et al., 1998; Rimet and Bouchez, 2012a). Identifications were carried out using Krammer and Lange-Bertalot (1991, 1997a,b, 2004) and nomenclature was unified using Algaebase (Guiry and Guiry, 2016).

Environmental variables (pH, conductivity) were measured in situ using instruments from the WTW company (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany), and transparency was measured using a Secchi disc. Total phosphorus (P_{tot}) and chlorophyll-a concentration were determined following standard methods (Hekera, 1999; Vernon, 1960).

2.2. Dataset construction and bioindication calculation

We combined four kinds of data for each sample: (i) taxon abundances at three different levels of taxonomic resolution; (ii) ecological variables delimiting trophic status; (iii) sample classification according to selected ecological parameters, and (iv) bioindication calculation.

(i) We generated three datasets of diatom relative abundance (percentage) for each sample: the first resolved to genus level (GL), the second resolved to species level but including unresolved species complexes (SLC), and the third to species level with cryptic species recognized (SL).

(ii) Total P (P_{tot}), transparency and chlorophyll-*a* concentration were selected as factors delimiting trophic gradients.

(iii) Each sample was placed in one of three habitat categories (A, B, C; see Supplementary Table S2) based on hierarchical clustering analysis against pH, conductivity, nutrient concentration and sediment type data (Hašler et al., 2008). Group A (19 localities) was represented by fishponds with low conductivity, pH > 8, high nitrogen or phosphorus concentration and bottom sediments with medium to high proportions of fine mud. Group B (21 localities) was represented by sites with high conductivity (above 500 $\mu S.cm^{-2}$), pH from 7.5 to 8.5, low nitrogen (2–2.5 $mg \cdot l^{-1}$) concentration and black organic bottom sediments. Group C (12 localities) was represented by dystrophic ponds (low conductivity, low pH) and sandy bottom sediments. Differences in P_{tot}, transparency and chlorophyll-a are shown in Supplementary Table S3.

(iv) The trophic index for each sample in the SLC dataset was calculated following Van Dam et al. (1994). We split the species matrix of the SLC dataset into two parts: species complexes (see Table 1) and remaining taxa. For each sample, mean weighed indicator values for trophy were calculated, based on either species complexes (SPC) or remaining taxa (RSP).

2.3. Data analyses

2.3.1. Influence of taxonomic resolution on composition patterns and their environmental correlates

Detrended correspondence analyses (DCA) were carried out to assess main (unconstrained) gradients in the datasets at different taxonomic resolutions (GL, SLC, SL). DCA was used because a preliminary test indicated that a unimodal approach was appropriate for the study (Šmilauer and Lepš, 2014). The species data were square-root transformed before analyses. Environmental factors (P_{tot}, transparency, chlorophyll-*a*, habitat categories) were correlated with the results of DCA to help with interpretation of the ordination results.

To test the direct effect of a selected environmental factor (P_{tot} as a proxy of trophic status) and habitat classification (habitat categories A, B, C) on species composition constrained ordination (Canonical correspondence analysis, CCA; Šmilauer and Lepš, 2014) was used, with each variable tested separately. The significance of each predictor was tested by a Monte Carlo permutation test with 999 permutations. Phosphorus (P_{tot}) was log-transformed before all analyses.

Finally, we used symmetric co-correspondence analysis (symmetric CoCA) to measure co-correspondence between pairs of matrices with different levels of taxonomic resolution. Co-correspondence analysis maximizes the weighted covariance between weighted averaged species scores of data sets. It thus attempts to identify patterns that are common to both data-sets (Schaffers et al., 2008; ter Braak and Schaffers, 2004). We used axis correlations, inertias (% model fit based on 4 axes), and *P* values based on the Monte Carlo permutational test with 999 permutations to interpret associations between pairs of matrices.

We then used partial CCA (pCCA) to explore the remaining (residual) variability in the SL and SLC matrices, after removal of variance attributable to the GL and SLC matrices in the CoCA. We controlled for the effects of coarse taxonomy in the CCA models by including coarse-taxonomy-derived WA site scores from four ordination axes in CoCA as covariates in pCCA. CoCA site scores reflect adjustments to maximize covariance between species matrices as described above (see Hanson et al., 2015; Šmilauer and Lepš, 2014). We tested the influence of P_{tot} and habitat classification separately using the Monte Carlo permutation test with 999 permutations. CANOCO 5 (ter Braak and Šmilauer, 2012) was used for DCA, (p)CCA and symmetric CoCA analyses.

Table 1
Cryptic species/species complexes recognized in our dataset.

Cryptic species	Abbrev.	Species complex (abbreviation)
Achnanthidium affine (Grunow) Czarn.	Achaff	*A. minutissimum* (Achagg)
Achnanthidium caledonicum (Lange-Bert.) Lange-Bert.	Achcal	
Achnanthidium eutrophilum (Lange-Bert.) Lange-Bert.	Acheut	
Achnanthidium jackii Rabenh.	Achjac	
Achnanthidium lineare W. Sm.	Achlin	
Achnanthidium minutissimum (Kütz.) Czarn.	Achmin	
Achnanthidium straubianum (Lange-Bert.) Lange-Bert.	Achstr	
Amphora ovalis (Kützing) Kützing	Ampova	*Amphora ovalis* (Ampagga)
Amphora pediculus (Kützing) Grunow ex A. Schmidt	Ampped	*Amphora pediculus* (Ampaggb)
Cocconeis pediculus Ehrenberg	Cocped	*Cocconeis pediculus* (Cocaggb)
Cocconeis placentula var. *euglypta* (Ehrenberg) Grunow	Cocple	*Cocconeis placentula* (Cocagga)
Cocconeis placentula var. *lineata* (Ehrenberg) van Heurck	Cocpll	
Cocconeis placentula var. *placentula* Ehrenberg	Cocplp	
Encyonema minutum (Hilse) D.G Mann	Encmin	*Cymbella ventricosa* (Cymagg)
Encyonema silesiacum (Bleisch) D.G. Mann	Encsil	
Eunotia bilunaris (Ehrenberg) Schaarschmidt	Eunbil	*Eunotia bilunaris* (*Eunagg*)
Fragilaria capucina var. *mesolepta (Rabenhorst) Rabenhorst*	Fracapm	*Fragilaria capucina* (Fraagga)
Fragilaria capucina var. *vaucheriae* (Kützing) Lange-Bertalot	Fracapv	
Fragilaria construens f. binodis (Ehrenberg) Hustedt	Fracob	*Fragilaria construens* (Fraaggb)
Fragilaria construens (Ehrenberg) Grunow *f. construens*	Fracoc	
Fragilaria construens f. exigua (W.·Smith) Schulz	Fracoe	
Fragilaria construens f. venter (Ehrenberg) Hustedt	Fracov	
Gomphonema parvulum (Kützing) Kützing var. *parvulum f. parvulum*	Gompar	*Gomphonema parvulum* (*Gomagg*)
Gomphonema exilissimum (Grunow) Lange-Bertalot & Reichardt	Gomexi	
Hippodonta capitata (Ehrenberg) Lange-Bertalot, Metzeltin & Witkowski	Hipcap	*Hippodonta capitata* (*Hipagg*)
Navicula cryptocephala Kützing	Navcry	*Navicula cryptocephala* (*Navagg*)
Nitzschia palea var. 1	Nitpal	*Nitzschia palea* (*Nitagg*)
Nitzschia palea var. *debilis* (Kützing) Grunow	Nitpad	
Nitzschia palea var. *palea* (Kützing) W. Smith	Nitpap	
Nitzschia paleacea (Grunow) Grunow	Nitpaa	
Planothidium lanceolatum (Brébisson ex Kützing) Bukhtiyarova	Plalan	*Planothidium lanceolatum* (*Plaagg*)
Sellaphora [pupula K-LB] ϕ small lanceolata	Selpupa	*Sellaphora pupula* (*Selagg*)
Sellaphora [pupula K-LB] ϕ tidy	Selpupb	
Sellaphora [pupula K-LB] ϕ tiny	Selpupc	
Sellaphora [pupula K-LB] ϕ caput	Selpupd	
Sellaphora [pupula K-LB] ϕ europa	Selpupe	
Sellaphora [pupula K-LB] ϕ grooved lanceolate	Selpupf	
Sellaphora [pupula K-LB] Φ slender	Selpupg	
Sellaphora pupula (Kützing) Mereschkovsky	Selpuph	
*Sellaphora pupula [K-LB]*ϕ 'spindle'	Selpupi	
Staurosirella pinnata (Ehrenberg) D.M. Williams & Round	Stapin	*Staurosirella pinnata* (*Staagg*)
Tabellaria flocculosa (Roth) Kützing	Tabflo	*Tabellaria flocculosa* (*Tabagg*)
Ulnaria ulna (Nitzsch) P. Compère	Ulnuln	*Ulnaria ulna* (*Ulnagg*)

2.3.2. Relationship between weighted averages of indicator values and measurements of water variables

Linear regression analysis was used to relate weighted averages of indicator values for trophic status (sensu Van Dam et al., 1994) to measured trophy (P_{tot}) using the SLC dataset split into two parts: species complexes (see Table 2) and remaining species. We assumed that species complexes (SPC) and remaining species (RSP) should indicate identical trophic gradients, i.e. their regression lines would be identical. To test this prediction, analysis of covariance (ANCOVA) was carried out and the effect of species category ("category") and its interaction with P_{tot} on average indicator values was tested using Statistica 12 package (Statsoft Inc., Tulsa, USA).

2.3.3. The predictive capacity of unresolved complexes and cryptic species

The predictive capacity of the species complexes and cryptic species to predict the species composition of the remaining assemblage was examined using predictive co-correspondence analysis (predictive CoCA; ter Braak and Schaffers, 2004). Models were created as follows: prediction of the remaining species assemblage with excluded species complexes by the species complexes partition, and by partition with resolved taxonomy, i.e. containing resolved cryptic species. In all cases, models were chosen with the number of axes corresponding to the highest prediction ability (% cross-validatory fit). Any fit above zero indicates that prediction is better than could have been expected by chance, implicitly validating the model. We always used the number of axes at which maximum prediction accuracy was obtained (ter Braak and Schaffers, 2004). Calculations were performed with the use of the "co-corresp" package (Simpson, 2009) in R (R Development Core Team, 2008).

2.3.4. Species responses to trophic gradients

Species-response curves for selected species complexes and their members (cryptic species) were modelled using generalized linear models (GLM). Due to overdispersion, presence–absence data were used and GLMs using the quasi-binomial distribution and logit-link function were calculated. Only species complexes that occurred in more than five sites were analysed. In addition, we excluded cryptic species with rare occurences (<5 sites) from the analyses. Phosphorus concentration (= trophic gradient, P_{tot}) was used as a predictor. Model complexity was evaluated using the Akaike Information Criterion statistic (AIC; Šmilauer and Lepš, 2014). Only significant response curves are reported. CANOCO 5 (ter Braak and Šmilauer, 2012) was used for GLM calculations.

2.3.5. Influence of taxonomic resolution on the identification of indicator species for habitat categories

We used the phi coefficient as a fidelity measure (De Cáceres and Legendre, 2009; Chytrý et al., 2002) to identify indicator (= diagnostic) taxa for the habitat groups (A, B, C). The indicator value of a taxon

Table 2
Results of DCA and CCA of matrices of diatom assemblages at three resolution levels (GL = genus level resolution, SLC = species level resolution with unresolved species complexes, SL = species level resolution with cryptic species recognized). In the CCA, the effect of each variable (P_{tot}, habitat category) was tested separately. Significance of the canonical axes was tested by the Monte Carlo permutation test with 999 permutations (**$P \leq 0.01$).

Matrix	DCA						CCA	
	Total variation	Eigenvalue		Length of gradient (SD)		Explained variation (first two axes; %)	Explained variation (all canonical axes; %)	Explained variation (first canonical axis; %)
		Axis 1	Axis 2	Axis 1	Axis 2		Habitat categories	P_{tot}
GL	2.53	0.28	0.17	2.7	1.9	17.6	8.1**	5.8**
SLC	7.14	0.49	0.34	3.8	2.9	11.7	6.2**	3.7**
SL	8.11	0.5	0.37	3.7	3.2	10.7	6.4**	3.8**

(phi × 100) varies from −100 to 100, and attains its maximum value when all individuals of a taxon occur in all samples of a single group, but not in any sample outside the group. In fidelity calculations, each sample in which the taxon is present is counted as an occurrence of the taxon, disregarding any abundance information. All groups were standardized to equal sizes (Tichý, 2002). The significance of the indicator value for each taxon was tested by the Fisher exact test at $P \leq 0.05$. Quality of delimitation was subsequently calculated as the average of positive fidelity values for all taxa of the respective group ("mean fidelity") and the number of diagnostic species in a group relative to its average taxonomic richness ("sharpness of classification"; Chytrý and Tichý, 2003). Mean fidelity statistics are high if many taxa have their occurrences concentrated in the group, and relatively low if the group includes mostly generalist species with broad ecological ranges. The sharpness index attains high values for groups with many diagnostic taxa with a high phi values (Chytrý and Tichý, 2003). Calculations were done using JUICE 7.0 (Tichý, 2002).

3. Results

A total of 263 species (SL matrix) belonging to 65 genera were identified. Diatom genera were unequally represented at the species level; *Gomphonema* was represented by 25 species, 28 genera were monospecific, and 17 genera were represented by 2 species. In all, 18 species complexes were represented by 43 cryptic species (Table 1).

3.1. Influence of taxonomic resolution on composition patterns and their environmental correlates

Separate DCA analyses of matrices at three taxonomic levels showed clear, similar patterns along the first two ordination axes (Fig. 1), suggesting that the main gradient along the first axis is strongly correlated with trophic status and also reflects the three habitat category classification (A, B, C), established on the basis of water chemistry and sediment type. As expected, total variation and gradient length increased (sharply) with increasing resolution from GL to SLC, but only slightly from SLC to SL (Table 2). Variation explained by the first two DCA axes dropped from ca. 18% for GL to ca. 12 and 11% for SLC and SL, respectively. Fine taxonomic resolution was responsible for decreased overlay and better separation of habitat categories along the first axis (Fig. 1). Explained variation (R^2) of site scores among habitat categories along the first DCA axis analysed by separate ANOVAs increased in the direction GL-SLC-SL, from 35% through 41% to 59%.

With GL resolution DCA, the more eutrophic part of the gradient is characterized by *Cyclotella*, *Cyclostephanos*, and *Lemnicola*. The less eutrophic part may be characterized by *Achnanthidium* (Fig. 1A). With SLC and SL resolution, *Lemnicola hungarica* and *Cyclotella meneghiniana* were the most typical species for the eutrophic part of the gradient. The less eutrophic part was represented by *Achnanthidium* and *Tabellaria* aggregates with SLC resolution (Fig. 1B), but by *A. lineare* and *A. caledonicum* with SL resolution (Fig. 1C).

Habitat categories and P_{tot} had significant marginal effects on species composition of all matrices, with slightly higher explained variation in the GL matrix (CCA; Table 2).

Cross-correlation between symmetric CoCA axes always yields coefficients above 0.979 for the first and second axes, and results of the Monte Carlo permutation test on the first CoCA axis' eigenvalue and on the sum of all eigenvalues (trace), representing total covariation in the data, were always significant (Table 3). This suggests that diatom assemblage matrices show almost identical multivariate patterns at three resolution levels.

We then used pCCAs to relate both SLC and SL to P_{tot} and habitat categories, using sample scores of coarser matrices derived from CoCA as covariables to control for variance attributable to GL or SLC (Supplementary Table S4). The adjusted variance explained by the SLC pCCA was very low (0.4 and 0.3% for P_{tot} and habitat categories, respectively) indicating that, after controlling for GL, most remaining variance in SLC was not related directly to the environmental variables in our models. The adjusted variance explained by the SL pCCA was also low but marginally significant in the case of habitat categories indicating that, after controlling for GL or SLC, some remaining variance in SL was related directly to the environmental variable (habitat groups) in our model (Table 3).

3.2. Relationship between trophic measures and diatom indication: comparison of species complexes and remaining species

Regression analysis of trophic indication based on the species complexes (SPC) and remaining species (RSP) on measured P_{tot} showed a close relationship between indicated and measured values for RSP ($R^2 = 0.32$, $P < 0.001$), but a weak relationship for SPC ($R^2 = 0.09$, $P = 0.032$). The slope of the regression for RSP bioindication was steeper than for SPC, indicating the higher sensitivity of RSP bioindication to changes in the phosphorus concentration (Supplementary Table S4, Fig. 2). SPC indication only matches RSP indication values above 0.4 $\text{ml} \cdot \text{l}^{-1}$ P_{tot}, below this SPC always indicated higher and mostly similar values compared to RSP (Fig. 2). The same pattern was found for chlorophyll-a, but a negative relationship for transparency (Supplementary Fig. S2).

3.3. The capacity for the unresolved and resolved species complexes to predict the species composition of the remaining assemblage

All predictive CoCA cross-validations gave results above zero, meaning that the predictive ability of the model was better than by chance. Predictive ability was, however, generally quite low; at most 1.4% of the matrix of remaining species was predicted by the cryptic species matrix (% cross validation; SL: 0.627%, 2 axes; SLC: 1.352%, 2 axes). The cryptic species matrix predicted the matrix of remaining species better (twice) than the unresolved complex species matrix did.

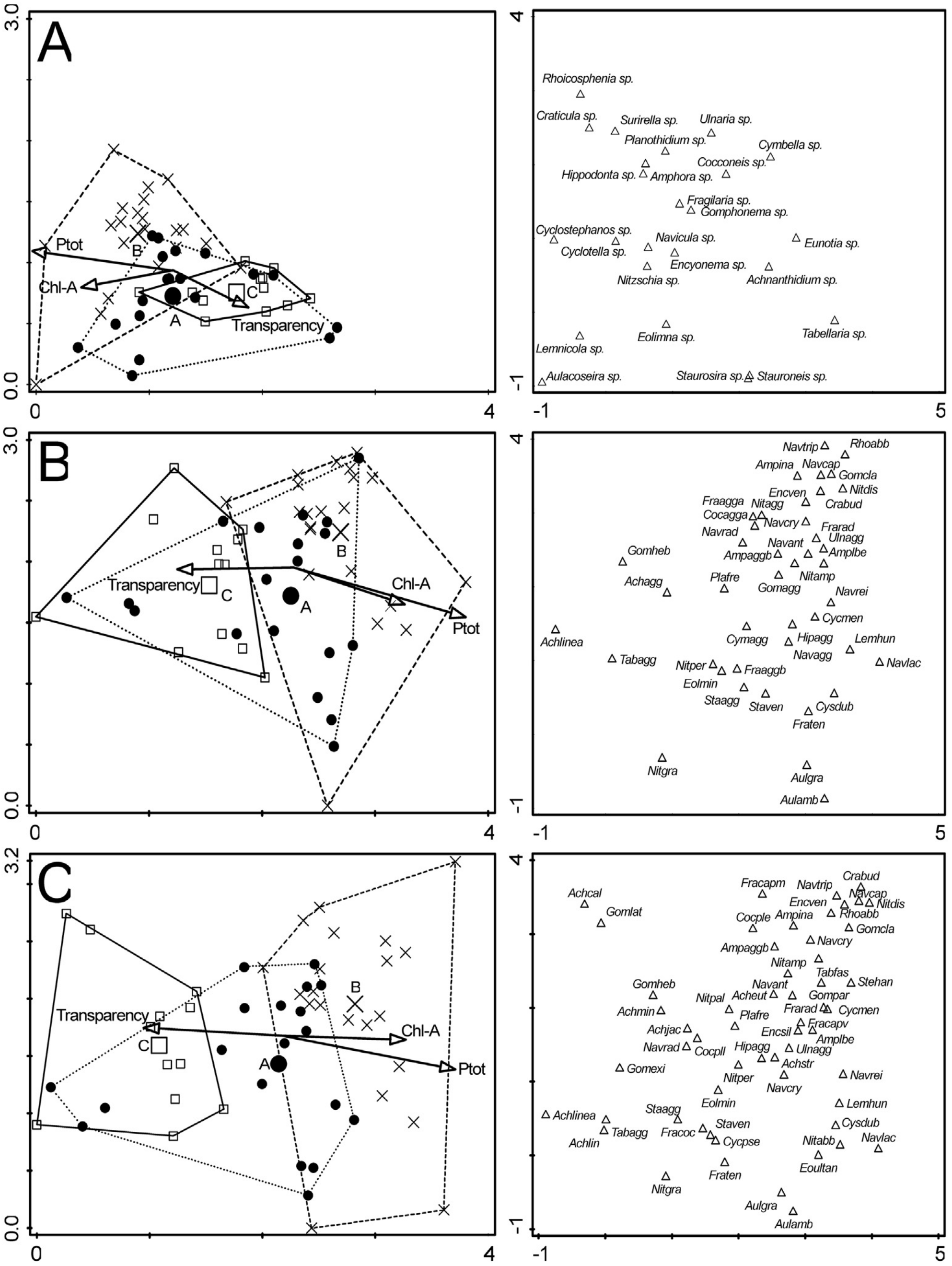

Fig. 1. Results of DCA analyses of matrices of diatom assemblages at three resolution levels (A: genus level resolution, B: species level resolution with unresolved species complexes, C: species level resolution with cryptic species recognized). Ordination diagrams represent first two unconstrained axes. Envelopes were drawn around members of each habitat category (A, B, C see Material and methods) and larger symbols represent centroids of the respective categories. Only taxa with at least 5% weight in the respective analysis are shown in the ordination diagram. Note reversed direction of the DCA diagrams of GL matrix along the first axis. Chl-A – chlorophyll *a*, Ptot – total phosphorus, Species abbreviations to complexes under study see Table 1. Other species: Rhoabb - *Rhoicosphenia abbreviata* (C. Agardh) Lange-Bertalot, Navtrip - *Navicula tripunctata* (O.F. Müller) Bory de Saint-Vincent, Ampina - *Amphora inariensis* Krammer, Gomcla - *Gomphonema clavatum* Ehrenberg, Nitdis - *Nitzschia dissipata* (Kützing) Grunow, Navcap - *Navicula capitatoradiata* Germain, Crabud - *Craticula buderi* (Hustedt) Lange-Bertalot, Frarad - *Fragilaria radians* (Kützing) D.M. Williams & Round, Navrad - *Navicula radiosa* Kützing, Navant - *Navicula antonii* Lange-Bertalot, Gomheb - *Gomphonema hebridense* W. Gregory, Gomlat - *Gomphonema lateripunctatum* Reichardt & Lange-Bertalot, Amplbe - *Amphora lange-bertalotii* var. *tenuis* Levkov & Metzeltin, Plafre - *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot, Nitamp - *Nitzschia amphibia* Grunow, Navrei - *Navicula reichardtiana* Lange-Bertalot, Cycmen - *Cyclotella meneghiniana* Kützing, Lemhun - *Lemnicola hungarica* (Grunow) F.E. Round & P.W. Basson, Achlinea - *Achnanthidium* cf. *linearoides*, Navlac - *Navicula lacuum* Lange-Bertalot, Hofmann, Werum & Van de Vijver, Nitper - *Nitzschia perminuta* (Grunow) M. Peragallo, Eolmin - *Eolimna minima* (Grunow) Lange-Bertalot & W. Schiller, Staven - *Staurosira venter* (Ehrenberg) Cleve & Moeller, Cycdub - *Cyclostephanos dubius* (Hustedt) Round, Fraten - *Fragilaria tenera* (W. Smith) Lange-Bertalot, Nitgra - *Nitzschia gracilis* Hantzsch, Aulgra - *Aulacoseira granulata* (Ehrenberg) Simonsen, Aulamb - *Aulacoseira ambigua* (Grunow) Simonsen, Tabfas - *Tabularia fasciculata* (C. Agardh) D.M. Williams & Round, Stehan -*Stephanodiscus hantzschii* Grunow, Cycpse - *Cyclotella pseudostelligera* Hustedt, Nitabb - *Nitzschia abbreviata* Hustedt, Eoultan - *Eolimna tantula* (Hustedt) Lange-Bertalot.

3.4. Examination of species complex and cryptic species responses to trophy

In most species complexes with frequent occurence within the dataset, cryptic species differed in trophic responses from their aggregate species and from each other (Table 4, Fig. 3). The three most frequent species complexes, *Gomphonema parvulum* agg., *Achnanthidium minutissimum* agg. and *Cocconeis placentula* agg. (occurring in 81–94% of samples), did not show a relationship with P_{tot} (null model; data not shown). Separate analyses of their members showed that cryptic species within each complex behaved contrastingly (Figs.

Table 3
Results of symmetric CoCA and partial CCA analyses of matrices of diatom assemblages at three resolution levels (GL = genus level resolution, SLC = species level resolution with unresolved species complexes, SL = species level resolution with cryptic species recognized). CoCA measures co-correspondence between pair of matrices with three different levels of taxonomic resolution. Axis correlations, inertias (% model fit based on four CoCA axes), and *p*-values based on Monte Carlo permutational test are reported. In partial CCA, the effect of each explanatory variable (P_{tot}, habitat categories) was tested separately on SLC and SL matrices. Coarse-taxonomy-derived WA site scores from 4 ordination axes in CoCA were used as covariates in the analyses (e.g. in GL-SLC, GL site scores from CoCA represent covariates and SLC represent dependent matrix). Significance of the canonical axes was tested by Monte Carlo permutation test with 999 permutations.

Matrix	Symmetric CoCA						Partial CCA (pCCA)			
	Total variation	Cross-correlation between CoCA axes		Explained variation (two first axes)	*P* value		Adjusted explained variation (%)	*P* value	Adjusted explained variation (%)	*P* value
		Axis 1	Axis 2		First axis	All axes	P_{tot}		Habitat categories	
GL-SLC	0.59	0.985	0.986	32.4	0.002	0.002	0.4	0.192	0.3	0.210
GL-SL	0.64	0.979	0.984	32.1	0.002	0.002	0.6	0.122	0.6	0.092
SLC-SL	1.78	0.999	0.999	24.8	0.002	0.002	0.2	0.294	0.7	0.052

3A-C, Table 4). Some cryptic species had increased probability of occurrence at oligotrophic (*G. exilissimum*, *A. lineare*, *A. caledonicum*) or eutrophic sites (*G. parvulum*, *A. eutrophilum*, *A. straubianum*). The most common, *A. minutissimum* sensu stricto, was frequent at sites with low and intermediate P_{tot}. *Cocconeis placentula* var. *placentula* had a unimodal response, with its optimum (and narrow tolerance) at lower P_{tot} (Table 4, Fig. 3C).

Nitzschia palea agg. was a less frequent (32%) complex and its probability of occurrence decreased slightly, but non-significantly, with increasing P_{tot}. *Nitzschia palea var. 1* behaved like the species complex, and because it was the most frequent cryptic species, strongly affected the response of the complex. The less frequent *N. palea* var. *palea* showed a unimodal response, with an optimum at higher P_{tot} concentrations (Fig. 3D, Table 4). *Fragilaria capucina* agg. and *Encyonema*/*Cymbella* agg. were frequent in the data set (60% and 56%, respectively), but neither the aggregates nor their members showed any relationship with P_{tot} (null model; data not shown). *Amphora ovalis* agg. and *Sellaphora pupula* agg. were too rare to allow statistical modelling.

3.5. Determination of indicator taxa for habitat categories

With fine taxonomic resolution, the number of indicator species for the habitat categories increased, sharply from GL to SLC, but only slightly from SLC to SL (Table 5). Mean fidelity was slightly higher in two of the three habitat categories (B, C) with coarse taxonomic resolution (GL), compared to SLC and SL resolution, which did not differ from each other. On the other hand, sharpness of classification increased with fine taxonomic resolution, especially between GL and SLC (Table 5). Only three aggregate species (*Fragilaria construens*, *Tabellaria flocculosa*, *Staurosirella pinnata*) but six cryptic species (*Fragilaria construens* f. *venter*, *Gomphonema parvulum*, *G. exilissimum*, *Achnanthidium caledonicum*, *A. affine*, *Encyonema minutum*) were identified as indicator species for the habitat categories.

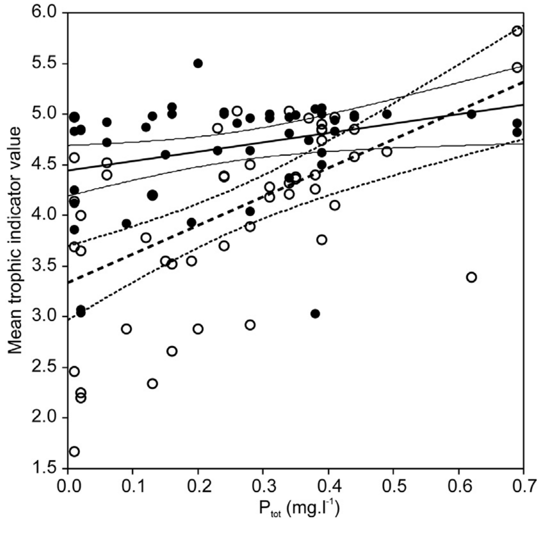

Fig. 2. Relationships between trophic indices calculated separately from RSP (empty circles) and SPC (full circles) and P_{tot} in 52 studied samples. For each species category (RSP, SPC), separate regression line with 95% confidence limits is shown (RSP: dashed lines, SPC: full lines).

4. Discussion

Over recent years biomonitoring of European aquatic ecosystems has been driven by the EU Water Framework Directive 2000/60/EC. Changes in "ecological status" are defined by the biotic response rather than by changes in environmental parameters (Birk et al., 2012), as for water quality monitoring worldwide (European-Commitee-for-Standardization, 2003; Kelly et al., 1998; Kusber, 2001; Lavoie et al., 2014; Schaumburg et al., 2004, 2005; Watanabe et al., 1988). Simultaneously, there have been major taxonomic revisions, including the recognition of cryptic species (Poulíčková et al., 2008b; Vanelslander et al., 2009).

4.1. Taxonomic resolution vs. assemblage composition and correlation with environment

Except for a few diatom indices based on genus level identification (Chessman et al., 1999, Rumeau and Coste, 1988; Wu, 1999), fine taxonomic resolution to species level is required by most diatom indices used in Europe (Coste et al., 2009; Lavoie et al., 2006; Rimet and

Table 4
Results of GLM testing the effect of P_{tot} on the probability of occurrence of selected cryptic species. The report provides the best model selected by the lowest AIC value with a test of the selected model against the null model based on a F statistic. Type represent model complexity (linear, quadratic), R^2 (%) provides a measure of explained variation, F test statistic and following P estimate of type I error rate correspond to an overall parametric test of the selected model against the null model. Only significant models are showed. Responses of all aggregate species to P_{tot} were not significant (not shown). In all models, a quasi-binomial model type with logit-link function on binarized response of species to log P_{tot} was used.

Species	Type	R^2 (%)	F	*P* value
Achnanthidium caledonicum	Linear	24.3	10.3	0.002
Achnanthidium eutrophilum	Linear	7.3	4.6	0.037
Achnanthidium lineare	Linear	7.9	4.3	0.043
Achnanthidium minutissimum	Linear	10.8	5.1	0.029
Achnanthidium straubianum	Linear	11.3	5.5	0.022
Gomphonema parvulum	Linear	22.9	12.3	0.001
Gomphonema exilissimum	Quadratic	20.0	6.2	0.004
Nitzschia palea var. 1	Linear	10.6	5.8	0.020
Nitzschia palea var. *palea*	Quadratic	18.3	4.5	0.016
Cocconeis placentula var. *euglypta*	Quadratic	10.6	3.8	0.030
Cocconeis placentula var. *placentula*	Quadratic	71.9	12.4	0.005

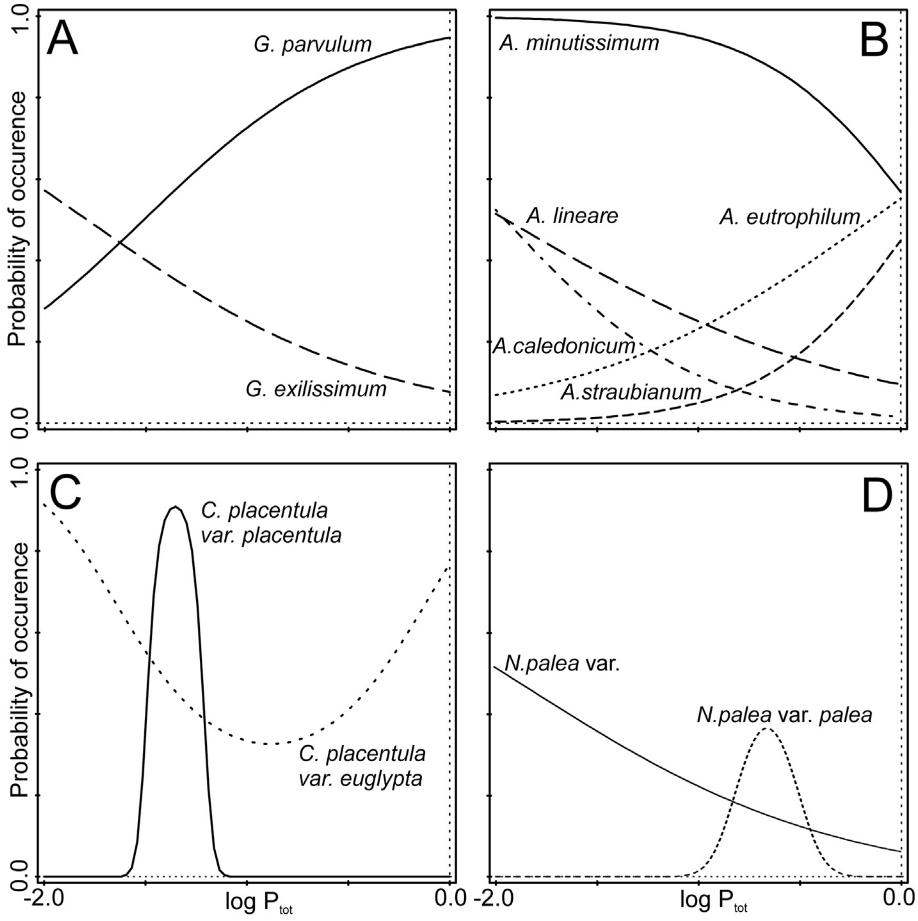

Fig. 3. Probability of occurrence for selected cryptic species as a function of log P_{tot} according to a generalized linear model (GLM, family = quasi-binomial, link = logit; see Table 4). All models are significant at $P \leq 0.05$. A – *Gomphonema* agg., B – *Achnanthidium minutissimum* agg., C - *Cocconeis placentula* agg., D - *Nitzschia palea* agg.

Bouchez, 2012a; Van Dam et al., 1994). However, increasing numbers of diatom species, described in a variety of publications, create problems for their use in routine analyses (Zampella et al., 2007). Some authors suggest that species sharing the same ecology/morphology should be grouped for biomonitoring purposes (DeNicola, 2000), whereas Rimet and Bouchez (2012b) compiled a check-list of diatom taxa with their allocation to life-forms, size classes and ecological guilds as a means to simplify biomonitoring. On the other hand, others see the future for biomonitoring in barcoding and new generation sequencing, producing taxonomic reference libraries for environmental barcoding (Zimmermann et al., 2014) or even in taxonomy-free approach (Apothéloz-Perret-Gentil et al. 2017).

Our results showed overall similarity in the efficiency of the three levels of taxonomic resolution for assessing the main environmental gradients in the data, suggesting that taxonomic resolution had little influence on matrix structure. The trophic gradient (total phosphorus, transparency and chlorophyll-a concentration) was the underlying factor behind the main gradient in the unconstrained analyses, and phosphorus also had a significant effect on assemblage composition in the constrained analyses, as frequently reported elsewhere (e.g. Poulíčková et al., 2004; Schönfelder et al., 2002). With fine taxonomic resolution heterogeneity in the dataset increased, and this increased total inertia and gradient length in unconstrained analyses (Schaffers et al., 2008), and slightly decreased predictability of the data sets (fit values) in the constrained analyses. More importantly, finer taxonomic resolution had no effect on the predictive merit of predictor factors but separated habitat categories better along the first ordination axis (Fig. 1). It is however clear that even genus level resolution seems sufficiently robust to describe the main gradients in our dataset, which may have important consequences for cost-efficiency decisions about particular methods (Growns, 1999). Previous comparisons of species or genus level bioassessment performance of diatoms (Growns, 1999; Hill et al., 2001; Chen et al., 2016; Raunio and Soininen, 2007; Rimet and Bouchez, 2012a; Wunsam et al., 2002) also showed the robustness and efficacy of genus level discrimination. Rimet and Bouchez (2012a) tried to explain this by the interaction of several factors, including the elimination of noise due to high proportions of extremely rare species, limited scope of studied environmental parameters, and difficulties around correct species identification.

Indeed, a certain proportion of diatom assemblages (up to 26%, Rimet and Bouchez, 2012a) can consitute extremely rare species, observed in only one sample (singletons). Low frequency, abundance, and/or narrow geographical range are generally considered as limiting factors, reducing a species' biomonitoring suitability (Diekmann, 2003), and such extremely rare species are usually eliminated from assessments (Lavoie et al., 2009, 2014). However, low abundance may be related to the specific habitat conditions to which the species is confined (Diekmann, 2003), and some authors therefore argue for the ecological importance of rare species (Potapova and Charles, 2004). Nevertheless, Lavoie et al. (2009) found that, after elimination of 40% of the rarest taxa, the indication power was still very good, suggesting that bioindications are fairly robust and resilient against incomplete sampling of taxonomic units (Ewald, 2003). The long tail of species with low abundance and/or frequency appears to represent large amounts of noise that may not be related to the studied environmental factors (Downes et al., 2000; Ewald, 2003), but caused by other factors operating locally or by chance (Diekmann, 2003). Indeed, our study showed that, with fine resolution, matrix heterogeneity increased when some

Table 5
Summary of the significant indicator taxa (species or genera) for three habitat categories (A, B, C), and evaluation of the quality of delimitation of the categories (mean fidelity, sharpness) for three matrices: GL = genus level resolution, SLC = species level resolution with unresolved species complexes, SL = species level resolution with cryptic species recognized. Indicator values represent phi × 100 coefficients and are based on presence/absence data. Only significant phi coefficients (with $P \leq 0.05$ in a Fisher exact test) are shown. Species aggregates and cryptic species are in bold. Species abbreviations see in Table 1 and Fig. 1. The rest of species: Navups - *Navicula upsaliensis* (Grunow) Peragallo, Diaten - *Diatoma tenuis* C. Agardh, Frabic - *Fragilaria bicapitata* (Mayer) D.M. Williams & Round, Calfal - *Caloneis falcifera* Lange-Bertalot, Genkal & Vekhov, Pinbor - *Pinnularia borealis* Ehrenberg var. *borealis*, Gomita - *Gomphonema italicum* Kützing, Gomoli - *Gomphonema olivaceum* (Hornemann) Brébisson, Nitdis - *Nitzschia dissipata* (Kützing) Grunow, Halven - *Halamphora veneta* (Kützing) Levkov, Surbre - *Surirella brebissonii* Krammer & Lange-Bertalot, Navtri -*Navicula trivialis* Lange-Bertalot, Plahol - *Platessa holsatica* (Hust.) Lange-Bert., Gomint - *Gomphonema intricatum* var. *vibrio* (Ehrenberg) Cleve, Eunarb - *Eunotia arcubus* Nörpel & Lange-Bertalot, Denten - *Denticula tenuis* Kützing, Fraten - *Fragilaria tenera* (W·Smith) Lange-Bertalot, Rospet - *Rossithidium petersonnii* (Hustedt) Round & Bukhtiyarova, Encvul - *Encyonema vulgare* Krammer var. *vulgare*, Gomacub - *Gomphonema acuminatum* var. *brebissonii* Kützing, Eunmin - *Eunotia minor* (Kützing) Grunow, Surbrek - *Surirella brebissonii* var. *kuetzingii* Krammer & Lange-Bertalot, Amppel - *Amphipleura pellucida* (Kützing) Kützing, Cymlbe - *Cymbella lange-bertalotii* Krammer, Gomauga - *Gomphonema augur* var. *augur*, Adamin - *Adalfia minuscula* (Grunow) Lange-Bertalot, Nitgra - *Nitzschia gracilis* Hantzsch, Encsub - *Encyonopsis subminuta* Krammer & E. Reichardt.

Category	Genus level resolution (GL)			Species level resolution with unresolved species complexes (SLC)			Species level resolution with cryptic species recognized (SL)		
	A	B	C	A	B	C	A	B	C
Mean taxon richness	18	16	17	29	26	27	31	27	29
Mean fidelity	1.2	7.0	10.9	2.0	4.7	8.2	2.0	4.6	8.9
Sharpness	2.6	11.6	18.9	8.3	17.3	28.4	9.0	18.0	32.5

Genus level resolution (GL)				Species level resolution with unresolved species complexes (SLC)				Species level resolution with cryptic species recognized (SL)			
EolimSp	45.8	–	–	Eolmin	42.3	–	–	Eolmin	42.3	–	–
CratcSp	–	50.4	–	Navups	37.0	–	–	Navups	37.0	–	–
RhoicSp	–	37.1	–	Diaten	33.3	–	–	Diaten	**33.3**	–	–
TryblSp	–	33.7	–	Frabic	33.3	–	–	Frabic	33.3	–	–
TabulSp	–	33.7	–	Calfal	33.3	–	–	Calfal	33.3	–	–
HalamSp	–	32.6	–	Pinbor	33.3	–	–	Pinbor	33.3	–	–
EunotSp	–	–	55.4	**Fraaggb**	**27.6**	–	–	**Fracov**	33.3	–	–
PlatsSp	–	–	50.0	Crabud	–	54.1	–	Crabud	–	54.1	–
RositSp	–	–	43.2	Navtrip	–	44.2	–	Navtrip	–	44.2	–
DentcSp	–	–	42.6	Gomita	–	41.5	–	Gomita	–	41.5	–
TabelSp	–	–	38.1	Gomoli	–	41.5	–	Gomoli	–	41.5	–
AmphpSp	–	–	34.3	Nitdis	–	37.2	–	Nitdis	–	37.2	–
StaurSp	–	–	33.9	Rhoabb	–	37.1	–	Rhoabb	–	37.1	–
StaurSp	–	–	30.2	Halven	–	36.8	–	Halven	–	36.8	–
–	–	–	–	Surbre	–	36.8	–	Subre	–	36.8	–
–	–	–	–	Navcap	–	35.6	–	Navcap	–	35.6	–
–	–	–	–	Tabfas	–	33.7	–	Tabfas	–	33.7	–
–	–	–	–	Amplbe	–	32.8	–	Amplbe	–	32.8	–
–	–	–	–	Navtri	–	29.5	–	**Gompar**	–	**31.1**	–
–	–	–	–	Plahol	–	–	50.0	**Gomexi**	–	–	**58.2**
–	–	–	–	Gomint	–	–	50.0	Plahol	–	–	50.0
–	–	–	–	Gomheb	–	–	48.2	Gomint	–	–	50.0
–	–	–	–	Eunarb	–	–	48.0	Gomheb	–	–	48.2
–	–	–	–	Denten	–	–	42.6	Eunarb	–	–	48.0
–	–	–	–	Fraten	–	–	41.7	**Achcal**	–	–	**45.3**
–	–	–	–	**Tabagg**	–	–	**38.1**	Denten	–	–	42.6
–	–	–	–	Rospet	–	–	35.0	Fraten	–	–	41.7
–	–	–	–	Encvul	–	–	35.0	**Encmin**	–	–	**40.5**
–	–	–	–	Gomacub	–	–	35.0	Tabagg	–	–	38.1
–	–	–	–	Eunmin	–	–	34.3	Gomacub	–	–	35.0
–	–	–	–	Surbrek	–	–	34.3	Rospet	–	–	35.0
–	–	–	–	Amppel	–	–	34.3	Encvul	–	–	35.0
–	–	–	–	Cymlbe	–	–	34.3	Gomauga	–	–	34.3
–	–	–	–	Gomauga	–	–	34.3	Surbrek	–	–	34.3
–	–	–	–	Adamin	–	–	34.3	Amppel	–	–	34.3
–	–	–	–	**Staagg**	–	–	**33.9**	**Achaff**	–	–	**34.3**
–	–	–	–	Nitgra	–	–	32.9	Eunmin	–	–	34.3
–	–	–	–	Encsub	–	–	32.4	Cymlbe	–	–	34.3
–	–	–	–	Staven	–	–	30.2	Adamin	–	–	34.3
–	–	–	–	–	–	–	–	Staagg	–	–	33.9
–	–	–	–	–	–	–	–	Nitgra	–	–	32.9
–	–	–	–	–	–	–	–	Encsub	–	–	32.4
–	–	–	–	–	–	–	–	Staven	–	–	30.2

highly abundant genera (e.g. *Gomphonema*) or complexes (e.g. *Sellaphora pupula* agg.) were replaced by numerous but low abundance, and rare cryptic species.

However, to explain strong co-correspondence between the matrices of various taxonomic resolutions, the respective genus and subordinate members (species, cryptic species) in the dataset must have similar ecological behaviour. This assumption stems from the idea that shared evolutionary ancestry can account for shared ecology (Losos, 2008). In diatoms, for example, some genera are known to have high trophic demands (*Stephanodiscus*), while others (*Aulacoseira*, *Cyclotella*) are representative of rather meso- or even oligotrophic waters (Krammer and Lange-Bertalot, 1991). Using phylogenetic tools, a recent study dealing with phylogenetic signal in diatom ecology (Keck et al., 2016) found several clades containing species that exhibited homogeneous ecology, especially for ions, inorganic nutrients (incl. phosphorus) and organic matter. There are, however, many studies on various organisms providing evidence that ecological similarity and phylogenetic relatedness are unrelated (see Losos, 2008 for review; Heino and Soininen, 2007). Moreover, we showed, in line with several recent papers (Poulíčková et al., 2008b; Vanelslander et al., 2009; Kulichová and

Fialová, 2016), that cryptic species within many species complexes exhibit niche differentiation despite their close phylogenetic relatedness (see below). This finding also explains why we found that, after controlling for coarse taxonomic matrices (GL or SLC), some remaining variance in the fine taxonomic resolution matrix (SL) was directly related to the environmental variable (habitat groups). Hence, ignoring the existence of cryptic species by using coarse taxonomic resolution might cause the loss of ecological information (Heino and Soininen, 2007) and obscure potentially suitable indicator cryptic species.

Alternatively, similar efficiency of different taxonomic resolutions in the assessment of community responses to environmental drivers can occur when, for example, each genus contains a small number of species (or is even monospecific), which decreases the chance of differential species responses (Growns, 1999). In our dataset, 43% of the genera were represented by a single species and 69% by less than three species. Chen et al. (2016) and Growns (1999) noted analogous situations with their datasets. Indeed, in a literature review of various organismal types, including algae and random simulations on various marine mollusc assemblages, Bevilacqua et al. (2012) showed that the level of species aggregation (i.e., higher taxa to species ratio) rather than taxonomic relatedness is important for the different levels of taxonomic resolution to be effective in detecting community responses to environmental variation. Intermediate values of genus/species ratio (SLC: 0.28, SL: 0.25) and the marked environmental gradient found within our dataset support Bevilacqua's analysis, showing the comparable effectiveness of higher taxonomic rank (genus) in depicting the community reponse to environmental variations under particular conditions.

However, defining a higher taxonomic level a priori may be unrewarding because it frequently restricts inferences about the causality of community patterns (Bevilacqua et al., 2013). Bevilacqua et al. (2013) therefore recommend mixing surrogates (taxonomic, phylogenetic, functional, etc.) and prefer the choice of ecologically meaningful groupings. Retention of greater (species level) ecological information has been recommended in the cases of easily recognizable indicator taxa or sensitive species (Bevilacqua et al., 2012). Species level identification is still supported by many diatomists (Jüttner et al., 2013; Kociolek, 2005; Patrick and Palavage, 1994; Ponader and Potapova, 2007). Thus, using a more relaxed approach with different surrogate types, which includes (cryptic) species level identification, particularly for big genera with known wide ecological amplitude and common occurence, such as *Navicula*, *Pinnularia*, *Gomphonema*, and *Nitzschia*, could improve the interpretation of statistical analyses. We can demonstrate this phenomenon using two examples.

A recent multivariate analysis (Chen et al., 2016) showed a moisture gradient in peatlands, with the genus *Pinnularia* appearing typical for relatively dry places. However, this is one of the most species-rich raphid diatom genera, with 730 accepted taxa (Guiry and Guiry, 2016). It is considered a cosmopolitan genus, occurring in a broad range of freshwater habitats, particularly with low electrolyte content (Round et al., 1990). However, in interpreting its position in ordination space as correlated with decreasing moisture (Chen et al., 2016), it is important to remember that one of its species complexes, *Pinnularia borealis*, is aerophytic, unlike many other *Pinnularia* species. Similarly, our recent results (Fig. 1) showed that the eutrophic part of the DCA ordination space was characterized by *Cyclotella*. This genus currently has 133 accepted taxa (Guiry and Guiry, 2016) and occurs in the plankton of standing waters, preferably with high electrolyte content (Round et al., 1990). The presence of centric diatoms on macrophytes can be explained by sedimentation from the plankton, and the presence of planktonic species in the epiphyton increases in plankton-rich eutrophic waters (Poulíčková et al., 2004). However, *Cyclotella* species frequently occur in mesotrophic waters, so when interpreting the position of *Cyclotella* in ordination space, it is important to be aware that *Cyclotella meneghiniana*, found in our samples (Fig. 1), tolerates eutrophic waters. Thus knowledge of species composition can help with the interpretation of results and extrapolations from particular datasets.

4.2. Indicator value of species complexes versus remaining species

Although cryptic diversity has recently been observed in many diatom species (Degerlund et al., 2012; Kaczmarska et al., 2014; Kulichová and Fialová, 2016; Poulíčková et al., 2010), there is still a broad gap in understanding the distribution, geography and ecological demands of cryptic species. Indeed, most floristic and ecological papers do not recognize cryptic species, because their identification is very difficult or even impossible without molecular data or special techniques (geometric morphometry, DAPI staining; Kulichová and Fialová, 2016; Poulíčková et al., 2016). However species complexes represent a considerable portion of periphytic assemblages (Supplementary Table S1; Kollár et al., 2015), most of which have broad ecological tolerances or no apparent optima (Poulíčková et al., 2008b; Schlick-Steiner et al., 2007). Our results showed that trophic bioindication based on a reduced diatom dataset (after removal of species complexes) closely followed the measured trophic gradient, while that based on a dataset comprising species complexes only was weakly related to trophic gradient. This was corroborated by non-significant responses of species complexes to phosphorus in our GLM analyses. Such weak indication-environment associations suggest that the indicator values of the majority of species complexes are unreliable. This may be explained simply by the ecological heterogeneity of their members that together give the broad tolerance of species complexes. Incorporating indicator values of species complexes into the calculation of weighted mean indicator values of the whole assemblage may in some cases result in worse environmental calibrations, especially when species complexes dominate the assemblage.

4.3. Species complexes versus their members: towards improving our knowledge of their ecology and environmental prediction

We found that, while most species complexes were poor indicator species for habitat categories, incorporating knowledge about cryptic diversity led to an increase in the number of indicative cryptic species, and also increased the classification precision (Table 5). This suggests that ecological heterogeneity exists within species complexes (De Cáceres and Legendre, 2009, Chytrý et al., 2002). However, inspection of each species complex within our dataset revealed more complicated patterns, and species complexes within our dataset (Table 1) could be divided into three types: 1. cryptic species with indistinguishable frustule morphology, lacking keys for identification, and/or with limited information on their distribution and ecology (e.g. *Navicula cryptocephala* agg.). These are worthy of future attention and investigation to fill the information gap, and to allow them to be transferred into one of the following groups: 2. cryptic species with low potential for improving indicative power due to shared ecology, or without a response to the studied gradient (e.g. *Fragilaria capucina* agg., *Encyonema*/*Cymbella* agg.); 3. morphologically (LM) more or less distinguishable cryptic species that are ecologically differentiated (*Achnanthidium minutissimum* agg., *Gomphonema parvulum* agg., *Nitzschia palea* agg.), with high potential for use in biomonitoring. These types are discussed below.

(1) The first type of “unresolved” species complex is represented by *Navicula cryptocephala*/*lothargeitleri* agg., which is more frequent in the epipelon than in the epiphyton (Supplementary Fig. S1; Poulíčková et al., 2010, 2016). These species are pseudocryptic, having extremely similar valve morphologies but distinguishable cytologically and using geometric morphometry (Poulíčková et al., 2016). Their autecology and geographical distribution are not yet understood, because neither technique for their identification (interphase nuclei structure, geometric morphometry) is routinely used in biomonitoring. The new species, *N. lothargeitleri* (Poulíčková et al., 2016), seems to occur rarely, but sympatric occurrence with *N. cryptocephala* has been recorded (Poulíčková et al., 2016). The routine use of barcoding may reveal the ecological

potential of this complex. Some progress in discriminating cryptic species has been made in cases such as *Nitzschia palea* agg. (Trobajo et al., 2009), *Eunotia bilunaris* agg. (Vanormelingen et al., 2008), *Fragilaria* and *Staurosira* (Schmidt et al., 2004). The unusual response of *Cocconeis placentula* var. *euglypta* (member of *Cocconeis placentula* agg.), with a high probability of occurrence in both low and high P concentrations (Fig. 3C) might mean that it still includes two, ecologically differentiated, entities. *Sellaphora pupula* agg. also plays an important role locally in the epipelon (Supplementary Fig. S1), but further investigations are necessary because its biogeography and autecology are unknown. Although it is rare in Czech ponds in general, this complex is common, particularly in British lakes (40% of epipelic assemblages in UK, 3% in the Czech and Hungarian ponds, Poulíčková et al., 2008b, 2014; Mann et al., 2004). Some cryptic species within this complex have been confirmed by molecular methods (Evans et al., 2008, 2009; Vanormelingen et al., 2013) and some of the morphospecies seem to be ecologically differentiated (Poulíčková et al., 2008b). Molecular data, autecology and identification criteria are still lacking for *Amphora pediculus* agg., *A. ovalis* agg., *Cocconeis pediculus* agg., *Hippodonta capitata* agg., *Planothidium lanceolatum* agg., *Staurosirella pinnata* agg., *Tabellaria flocculosa* agg., and *Ulnaria ulna* agg.

(2) Although the second type of species complex occurs frequently in our dataset, we cannot use them for biomonitoring trophic status, because neither the complex nor its members show any relationship with P_{tot} concentration. This does not mean that they cannot be indicators of other gradients (e.g. pH, moisture). *Encyonema silesiacum* and *E. minutum* (previously grouped under *Cymbella ventricosa*) have overlapping sizes (*E. silesiacum*: length 16–42 μm, breadth 5.9–9.6 μm; *E. minutum*: 7–23 μm and 4.2–6.9 μm) but can be distinguished by their stria density (*E. silesiacum* 11–14 per 10 μm, *E. minutum* 15–18 per 10 μm) (Hofmann et al., 2013). Although the species are distinguishable and quite frequent, they do not seem to be promising for trophic status assessment, like *Fragilaria capucina* agg.

(3) The third type of species complex seems promising due to the morphological variation and ecological differentiation of its components. *Achnanthidium minutissimum* agg. is a common species complex (Rimet and Bouchez, 2012a), often abundant in the epiphyton and epilithon (Supplementary Fig. S1) of both lentic and lotic waters (Almeida et al., 2014; Kollár et al., 2015). Although mostly reported as an indicator of good water quality (Almeida et al., 2014; Feio et al., 2007), it seems to be highly tolerant to metal contamination and natural disturbance (Cantonati et al., 2014; Kovács et al., 2006). This complex consists of 18 entities (varieties according to Omnidia software v. 5.3.; Lecointe et al., 1993) which are morphologically difficult to identify (Coste et al., 2009). New species have even been described from the Antarctic region (Van de Vijver and Kopalová, 2014). Using a refined identification key based on samples from similar geographical area (Wojtal et al., 2011) we were able to distinguish seven morphospecies. *Achnanthidium minutissimum* agg. did not show any relationship to trophic gradient (P_{tot}), but three of the cryptic species, *A. minutissimum*, *A. lineare*, *A. caledonicum*, were characteristic for lower trophic levels (Fig. 1B, C). This was also supported by the GLM models (Fig. 3B). The most frequent cryptic species, *A. minutissimum*, strongly influenced the response of the whole complex. Two other cryptic species, *A. eutrophilum* and *A. straubianum*, show positive relationships to higher P_{tot} concentrations, but are less frequent. The other cryptic species are extremely rare. Only two *A. minutissimum* agg. members (*A. caledonicum*, *A. affine*) can be considered as indicative (diagnostic) species in our dataset (Table 5). Based on our results and literature data (Potapova and Hamilton, 2007), we recommend paying attention to this complex in future studies and biomonitoring.

Gomphonema parvulum agg. is present in >51% river samples (Rimet and Bouchez, 2012a) and occurred in 88% of our samples. This is a morphologically highly variable diatom (Rose and Cox, 2014) that occurs in a wide range of water qualities (Almeida et al., 2014; Krammer and Lange-Bertalot, 1997a,b). Based on both molecular and morphological data (Abarca et al., 2014; Kermarec et al., 2013), at least four taxa have been discriminated. Life history studies (Rose and Cox 2013, 2014) have demonstrated the distinctiveness of strains within this species, and highlight its taxonomic complexity. We were able to distinguish two cryptic species in the epiphyton of Czech fishponds that were significantly differentiated with respect to phosphorus concentration. *Gomphonema parvulum* showed positive, and *G. exilissimum* negative, relationships to P_{tot} (Figs. 1C, 3C), which is in agreement with previous records (reviewed by Jüttner et al., 2013). They are morphologically very similar and overlap significantly in size, requiring careful observation of each sample (*G. parvulum*: length 10–36 μm, breadth 5–8 μm, stria density in 10 μm 7–20; *G. exilissimum*: length 20–38 μm, breadth 4.5–6 μm; stria density in 10 μm12–14) (Hofmann et al., 2013). Moreover, Jüttner et al. (2013) described another morphologically similar species, *G. varioreduncum* Jüttner, Ector, E. Reichardt, Van de Vijver & E.J. Cox, typical of slightly to moderately acidic, usually oligotrophic waters (Sweden, Wales). If this taxon is misidentified as *G. parvulum*, the sites would be misclassified in the ecological status assessments (Jüttner et al., 2013). This complex would benefit from further investigation, including the application of barcoding techniques.

The diatom species complexes we discuss (Table 1) are the most frequent in our dataset, representing epiphytic communities in shallow mesotrophic and eutrophic ponds. Other diatom complexes can be found in the plankton (Degerlund et al., 2012; Kaczmarska et al., 2014), peat bogs and acidic waters (Kulichová and Fialová, 2016, Vanormelingen et al., 2008), and in oligotrophic lakes (Schmidt et al., 2004). We have tried to point out those aspects that need to be taken into account in contemporary ecological studies. We believe that investigations into cryptic diversity, including morphological and molecular approaches, have the potential to overcome recent limits and should improve bioassessment methods in the future.

5. Conclusions

Diatom epiphytic assemblages are dominated by species complexes that consist of different number of cryptic species. Bioindication based exclusively on species complexes showed lower sensitivity to the phosphorus gradient than the reduced dataset without these complexes. Fine taxonomic resolution was responsible for increasing the heterogeneity of the dataset, increasing the number of indicator species for habitat categories, and better separation of habitat categories in the ordination space. Within most frequently occurring species complexes, cryptic species differed in their responses to trophy from their complexes and from each other. Fine taxonomic resolution is recommended for cases where the assemblage is dominated by good indicator, easily recognized cryptic species. Thus research into the autecology of cryptic species is worth future study.

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Appendix A. Supplementary data

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5. CONCLUSIONS

5.1 Species richness and composition of epiphytic diatom communities

Diatoms form complex and structured films on plant substrate (Fig. 1-6 in paper II, Fig. 6 in paper III). Significant differences were found in the diversity of different ecoregions and water types - lotic/lentic. Altogether, 131 taxa were found in 25 fishponds and 13 small streams located in the Svitava River Basin and the White Carpathian Mountains in the Czech Republic (Kollár et al., 2015). In the meso-oligotrophic alpine Lake Valagola 78 taxa were identified (Letáková et al., 2016). And finally 263 taxa were found in the study focused on ponds of various characteristics located throughout the Czech Republic (Poulíčková et al., 2017), where species diversity varied from 11 to 51 taxa. The most species-rich place was the dystrophic Novozámecký Pond, the smallest taxon amount was found in the highly eutrophied Staňkovský Pond. The dominant epiphytic diatom *Achnanthidium minutissimum* agg. (Kollár et al., 2015; Poulíčková et al., 2017) was able to create even 88% of the community (Kollár et al., 2015). The other two most frequently occurring species/aggregates (Kollár et al., 2015; Poulíčková et al., 2017) were *Gomphonema parvulum* agg. with representation 1–48% and *Cocconeis placentula* Ehrenberg with representation 1–100% (Kollár et al., 2015). Species complexes represented the majority of epiphytic assemblages but this dominance did not lead automatically to reduction of overall diversity of the sample. *Fragilaria polonica* M. Witak & Lange-Bertalot and *Staurosirella pinnata* (Ehrenberg) D. M. Williams & Round were the most common species in Lake Valagola (Letáková et al., 2016).

5.2 Epiphytic diatoms and ecological parameters

CCA analysis showed significant influence of pH and water streaming – running/stagnant water (Kollár et al., 2015). Species correlating with higher pH were *Amphora pediculus* (Kützing) Grunow, *Cocconeis pediculus* Ehrenberg, *Cymbella excisiformis* Krammer, *Encyonopsis cesatii* (Rabenhorst) Krammer, *Encyonopsis microcephala* (Grunow) Krammer, *Eunotia arcus* Ehrenberg, *Gomphonema pumilum* (Grunow) E. Reichardt & Lange-Bertalot, *Nitzschia palaeformis* Hustedt and *Nitzschia sinuata* (Thwaites) Grunow, lower pH was preferred by *Mayamaea atomus* (Kützing) Lange-Bertalot, *Planothidium ellipticum* (Cleve) M. B. Edlund, *Planothidium lanceolatum* (Brébisson ex Kützing) Lange-Bertalot and *Nitzschia palea* (Kützing) W.Smith. Typical for lentic waters were diatoms like *Fragilaria brevistriata (recently accepted name Pseudostaurosira brevistriata* (Grunow) D.M. Williams & Round), *Encyonopsis microcephala* (Grunow) Krammer, *Eunotia arcus* Ehrenberg, *Denticula tenuis* Kützing, lotic waters often contained *Cocconeis pediculus* Ehrenberg, *Gomphonema angustatum* (Kützing) Rabenhorst and *Navicula tripunctata* (O. F. Müller) Bory.

Regression analysis confirmed that epiphytic diatom assemblages reflect trophic gradient, although there are significant differences in the relationship of species complexes and remaining taxa to phosphorus concentrations (Poulíčková et al., 2017). Oligo or mesotrophic waters were preferred by *Achnanthidium minutissimum* sensu stricto (Kützing) Czarnecki, *Achnanthidium lineare* W. Smith, *Achnanthidium caledonicum (Lange-Bertalot) Lange-Bertalot, Gomphonema exilissimum* (Grunow) Lange-Bertalot & Reichardt. Eutro or hypertrophic waters were correlated with *Achnanthidium eutrophilum* (Lange-Bertalot) Lange-Bertalot, *Achnanthidium straubianum* (Lange-Bertalot) Lange-Bertalot, *Gomphonema parvulum* (Kützing) Kützing.

The spatial distribution of epiphytic diatoms is also influenced by host plant distribution (Letáková et al., 2016).

5.3 Substrate specificity problematics and implications to biomonitoring

The influence of the substrate on the composition of the epiphytic community did not show uniform results in investigated localities. No significant specificity was noted in the

Czech ponds and small streams except *Lemna minor* L. Species such as *Fragilaria brevistriata (Pseudostaurosira brevistriata), Staurosirella pinnata* or *Nitzschia palaeformis* avoid *Lemna minor* as a host plant. *Lemnicola hungarica* (Grunow) Round & Basson, a diatom typical for *Lemna minor*, inhabited also *Phragmites australis* (Cav.) Steud in the Svitava River basin (Kollár et al., 2015). In case of the oligo-mesotrophic Lake Valagola, significant differences were found in diatom species richness, diversity and composition between the two main host plants. *Potamogeton gramineus* L. assemblages were characterized by higher species richness and diversity, and by the large-celled, adnate diatom species *Epithemia adnata* (Kützing) Brébisson, *Rhopalodia gibba* (Ehrenberg) Otto Müller, *Eunotia arcus* Ehrenberg and *E. arcubus* Nörpel & Lange-Bertalot. *Chara aspera* Willd was preferred by the small-celled, motile diatom species *Brachysira neoexilis* Lange-Bertalot and *Encyonopsis cesatii* (Rabenhorst) Krammer (Letáková et al., 2016).

It has been proved then, that epiphytic diatoms are suitable for bioindication in generally more nutrient rich Czech fishponds and rivers, as their distribution is not biased by plant substrate type. Although *Lemna* sp. should be excluded from the macrophytes used for this purpose.

5.4 Proportion of species complexes within epiphytic assemblages

Surprisingly, species complexes represented the majority of epiphytic assemblages (up to 97%) with no significant differences between lotic and lentic waters or substrates. In the case of pond epiphyton (Poulíčková et al., 2017), 18 species complexes were formed by 43 cryptic species and created 3-97% of the community (average 56,81 ± standard deviation 25,19 %). The most common was the complex of *Achnanthidium minutissimum* agg. (with the following morphotypes: *Achnanthidium affine* (Grunow) Czarnecki, *Achnanthidium caledonicum* (Lange-Bertalot) Lange-Bertalot, *Achnanthidium eutrophilum* (Lange-Bertalot) Lange-Bertalot, *Achnanthidium jackii* Rabenhorst, *Achnanthidium lineare* W. Smith, *Achnanthidium minutissimum* (Kützing) Czarnecki and *Achnanthidium straubianum* (Lange-Bertalot) Lange-Bertalot. Even though the research in Valagola was focused in a different way, out of these 18 species complexes, *Achnanthidium minutissimum, Amphora pediculus, Cocconeis placentula* and *Gomphonema parvulum* species complexes have been noticed.

5.5 Species complexes indicatory power and taxonomic resolution in biomonitoring

Precise taxonomical identification did not play such an important role in biomonitoring as could have been expected. Simple genus level identification of epiphytic diatoms brought sufficient information required for normal routine biomonitoring (Poulíčková et al., 2017). However, fine taxonomic resolution (with resolved cryptic species) still showed sufficient variance related to the environmental variable (habitat groups), and increased the sharpness of classification, number of indicator species for habitat categories, and gave better separation of habitat categories in the ordination space. Species complexes showed lower sensitivity to changes in phosphorus concentration than the rest of species, thus seem to have low indicatory power in trophic state bioassassment (Poulíčková et al., 2017).

5.6 Species complexes of epiphytic diatoms with potential to improve monitoring

Ecological heterogeneity exists within species complexes. Species complexes of epiphytic diatoms might be divided into three groups: 1. cryptic species with indistinguishable frustule morphology, lacking keys for identification, and/or with limited information on their distribution and ecology (e.g. *Navicula cryptocephala* agg.). These are worthy of future attention and investigation to fill in the information gap, and to allow them to be transferred into one of the following groups: 2. cryptic species with low potential for improving indicative power due to shared ecology, or without a response to the studied gradient (e.g. *Fragilaria capucina* agg., *Encyonema/Cymbella* agg.); 3. morphologically (LM) more or less distinguishable cryptic species that are ecologically differentiated (*Achnanthidium minutissimum* agg., *Gomphonema parvulum* agg., *Nitzschia palea* agg.), with high potential for use in biomonitoring (Poulíčková et al, 2017).

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DIVERSITY AND DISTRIBUTION OF EPIPHYTIC DIATOMS

Summary of Ph.D. Dissertation

Supervisor: Prof. RNDr. Aloisie Poulíčková, CSc.

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This Ph.D. thesis was carried out within the framework of internal Ph.D. studies at the Department of Botany, Faculty of Science, Palacký University Olomouc during the years 2013-2018.

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The Ph.D. Thesis is available in the Library of Biological Department of Faculty of Science at Palacký University, Olomouc – Holice, Šlechtitelů 27.

Prof. Ing. Aleš Lebeda, DrSc.

Chairman of the Commission for the Ph.D.

Theses for Study Subject Botany

Faculty of Science, Palacký University

CONTENT

1. INTRODUCTION

Diatoms are unicellular autotrophic organisms, belonging into the group of Stramenopiles (Adl et al., 2012). Their typical feature is a silica cell wall – a frustule. They appear all over the world in various aquatic or wet terrestrial habitats. Traditionally they have been divided into two groups according to the valve symmetry: to *centrics* with radial symmetry and *pennates* with bilateral symmetry.

Diatoms adapt to the environment and biotic influences forming different life strategies. They are classified in various ways according to different authors. Mann et al. (2016) divides diatoms into three groups: they live either suspended, attached or they are motile. The latter two, living in the association with surface, are often called benthic. Benthic diatoms are a diverse group of species living close to the substrate, they include motile and non-motile species. Every type of surface is very specific and diatoms are usually divided according to this. Benthic algae are divided into: epipelon, endopelon, epipsammon, endopsammon, epilithon, endolithon, epixylon, epizoon, endozoon, endophyton and finally epiphyton (reviewed by Poulíčková et al., 2008).

Epiphytic diatoms are a special diatom group that lives in asssociation with plant material. Diatoms can live either inside – *endophytic*, or on the surface – e*piphytic*, creating sometimes very huge extensive colonies. For a detailed introduction of epiphytic diatoms see Paper I.

1.1 Paper I

Ecology and applications of freshwater epiphytic diatoms – review

Markéta Letáková, Markéta Fránková & Aloisie Poulíčková

Abstract

Epiphytic diatoms perform a variety of ecological functions. Diatoms are important primary producers and sources of oxygen which can modify the chemistry of the surrounding aquatic environment. They may live attached to plant surfaces with the help of extracellular polymeric substances and compete with plants for resources (e.g., light, nutrients). Thus, they represent an excellent model system for studies on interactions between epiphytes and their host plants under different environmental conditions. Further, the practical usage of epiphytic diatoms in biomonitoring begs questions concerning substrate specificity, diatom biodiversity, and species delimitations. This review focuses on specific aspects of freshwater epiphytic diatom ecology as adaptations for epiphytic way of life, plant-diatom relationships, and implications for biomonitoring.

Key words: epiphytic diatoms, ecology, substrate specificity, biomonitoring, species complexes

2. MAIN AIMS OF THE DOCTORAL THESIS

This dissertation focuses on the community of epiphytic diatoms in order to account for the knowledge about the life on the plant substrate and it tries to connect this with the practical aspects important in biomonitoring. At the centre of interest are the following aims:

- To investigate the structure, diversity and composition of epiphytic diatom communities.
- To study the influence of ecological parameters on the epiphytic diatoms composition.
- To try to summarize and clarify the problematics of the substrate specificity and its consequences for biomonitoring.
- To investigate the contribution of species complexes and cryptic/ semi-cryptic diatom species to overall epiphyton diversity.
- To compare how species complexes and other species follow the trophic gradient and to find out how the accuracy of diatom identification influences water monitoring.
- To point out epiphytic species complexes whose resolving has the potential to meliorate biomonitoring.

3. METHODS

3.1 Material, sampling and preparation

Epiphytic diatoms with their substrate (microphytes and macrophytes) were collected from altogether 79 localities. They were mostly represented by shallow fishponds and small local streams of the Czech Republic (Kollár et al., 2015; Poulíčková et al., 2017). For the Letáková et al. (2016) study, samples were taken in Lake Valagola in the Brenta Dolomites in Italy. Basic characteristics of each place are given in the individual papers.

Sampling always started when the submersed part of the plant was cut with the scissors and placed into a plastic container. In the laboratory, samples were worked out within a few hours. Macrophytes were cut into smaller pieces, they were placed in the Erlenmeyer flasks and filled with around 300 ml of 30% H_2O_2 (volume differed according to the amount of plant material) in order to start the slow oxidation. Everything was done with great attention to prevent contaminations. After several days, the samples were boiled until the volume decreased considerably, a small amount of $K_2Cr_2O_7$ and 1 ml of conc. HCl were added. In such a way the oxidation process was finished. The diatom samples were washed out in distilled water several times until they reached neutral pH. The appropriate concentration of clean diatom frustules was prepared, and permanent samples were mounted with Naphrax.

Diatoms were observed using a light microscope Zeiss 'Primo star'(Germany) and Zeiss Axioskop 2 (Zeiss, Jena, Germany) with phase contrast, and images were taken with an Axiocam digital camera. All the samples were investigated qualitatively and semi-quantitatively. For the later one, 400 diatom valves were counted within each sample. Diatoms were identified using the following diatom determination keys: Krammer, (2000, 2002, 2003), Lange-Bertalot (2001), Levkov (2009), Lange-Bertalot et al. (2011), Hofmann et al. (2013), Lange-Bertalot et al. (2017). Nomenclature has been unified according to AlgaeBase (Guiry & Guiry, 2015a; 2015b; 2016).

For SEM observation, clean diatom frustules or herbarized materials were mounted on aluminium stubs, coated with gold and observed in Scanning Electron Microscope Zeiss EVO 40 XVP Zeiss.

Fresh algal assemblages were observed on a stem epidermis using the LTM for ESEM without any pre-treatment. Samples were observed using the FEI ESEM QUANTA 650FEG with beam energy 20 kV, probe current 35 pA and working distance 8.5 mm.

3.2 Measuring ecological parameters

Ecological parameters including temperature, pH and conductivity were measured *in situ* using the WTW company instrument (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) or with a multiparametric Hydrolab, Transparency was measured using a Secchi disk. Major ions, main algal nutrients and chlorophyl a concentrations were determined following the standard methods (Vernon, 1960; Hekera, 1999; APHA, 2000).

3.3 Statistical analysis

Various multivariate statistical analyses were used in order to find the patterns of diatom distribution and factors influencing epiphytic diatom communities. Methods are described in detail in attached articles (Kollár et al., 2015; Letáková et al., 2016; Poulíčková et al., 2017).

4. RESULTS

4.1 Paper II

The low temperature method for Environmental Scanning Electron Microscopy – a new tool for observation of diatom assemblages *in vivo*

Markéta Fránková, Aloisie Poulíčková, Eva Tihlaříková, Vilém Neděla, Kateřina Šumberová & Markéta Letáková

Abstract

The Low Temperature method for sample stabilization in environmental scanning electron microscopy appears to be a promising tool for the observation of diatom assemblages *in vivo*. Use of the environmental scanning electron microscope, in comparison to the conventional scanning electron microscope, enables study of fresh material without any chemical pretreatment and conductive coating. The newly developed Low Temperature Method, introduced in this paper, offers higher resolution and better resistance of wet samples to radiation damage. We used natural epiphytic algal assemblages to image 3D structure of: i) biofilm/periphyton and its physical complexity, ii) diatoms with their extracellular mucilaginous secretions enabling cells to attach to the substrate, iii) diatom colony formation, and iv) intact diatom cells/frustules in contrast to separated empty valves observed in the conventional scanning electron microscope. This study demonstrates the potential of this new method for environmental scanning electron microscopy in diatom biology and ecology in comparison with other imaging methods.

Key words: *living diatoms, epiphyton, low temperature method for ESEM*

4.2 Paper III

Substrate specificity and fine-scale distribution of epiphytic diatoms in a shallow tarn in the Brenta Dolomites (south-eastern Alps)

Markéta Letáková, Marco Cantonati, Petr Hašler, Nicola Angeli & Aloisie Poulíčková

Abstract

The host-specificity of epiphytic diatom species has long been debated. Scuba divers sampled epiphytic diatoms in the shallow Alpine Lake Valagola (average depth c. 2 m) along seven transects (length: 30–144 m) in West-East direction. The bottom of the tarn was covered by macrophytes dominated by *Chara aspera* and *Potamogeton gramineus*. Factors affecting epiphytic-diatom spatial distribution at a fine scale were tested.

Dataset was tested using Redundancy Analysis (CANOCO package) and one-way ANOVA (NCSS package).

The analysis separated sampling sites into two groups: the tarn shore dominated by *Potamogeton gramineus*, and the central area dominated by *Chara aspera*. Diatom species richness, diversity, and composition differed significantly between the two main host plants. *Potamogeton gramineus* assemblages were characterized by higher species richness and diversity, and by the large-celled, adnate diatom species *Epithemia adnata*, *Rhopalodia gibba*, *Eunotia arcus* and *E. arcubus*. *Chara aspera* was preferred by the small-celled, motile diatom species *Brachysira neoexilis* and *Encyonopsis cesatii*.

The spatial distribution of epiphytic diatoms in the shallow, oligo-mesotrophic Lake Valagola is influenced by host plant composition and distribution. Epiphyton size structure suggests that *Chara* represents a less appropriate substrate for long diatoms.

Epiphyton, mountain lake, host specificity, epiphyton size structure, *Chara aspera*, *Potamogeton gramineus*, diatoms.

Key words – Epiphyton, mountain lake, host specificity, epiphyton size structure, *Chara aspera*, *Potamogeton gramineus*, diatoms.

4.3 Paper IV

Epiphytic diatoms in lotic and lentic waters – diversity and representation of species complexes

Jan Kollár, Markéta Fránková, Petr Hašler, Markéta Letáková & Aloisie Poulíčková

Abstract

Small streams and shallow ponds represent sensitive ecosystems and attached diatoms can serve as integrative indicator with fast response to environmental changes. Development of methods for ecological monitoring throughout Europe and their calibration for particular ecoregions are not finished yet and databases need to be filled by data from undersampled regions and overlooked substrates. The present study aims to explore the diversity of epiphytic diatoms in unexplored catchment areas with special attention to substrate specificity and distribution of unresolved diatom species complexes. Significant differences were found in diversity of both regions and water types (lotic/lentic). No significant differences were found in the case of substrates. CCA analysis showed significant influence of pH, water streaming (streaming/stagnant) and Lemna substrate to species composition. Surprisingly species complexes represent the majority of epiphytic assemblages with no significant differences between lotic and lentic waters or substrates except of Lemna. The high representation of complexes does not lead automatically to reduction of overall diversity of the sample.

Key words: diatoms, epiphyton, lotic and lentic waters, species complexes

4.4 Paper V

Species complexes within epiphytic diatoms and their relevance for the bioindication of trophic status

Aloisie Poulíčková, Markéta Letáková, Petr Hašler, Eileen Cox & Martin Duchoslav

Abstract

The popularity of aquatic bioassessments has increased in Europe and worldwide,with a considerable number of methods being based on benthic diatoms. Recent evidence from molecular data and mating experiments has shown that some traditional diatom morphospecies represent species complexes, containing several to many cryptic species. This case study is based on epiphytic diatomand environmental data from shallowfishponds, investigating whether the recognition and use of fine taxonomic resolution (cryptic species) can improve assessment of community response to environmental drivers and increase sharpness of classification, compared to coarse taxonomic resolution (genus level and species level with unresolved species complexes). Secondly, trophy bioindication based on a species matrix divided into two compartments (species complexes and remaining species) was evaluated against the expectation that species complexeswould be poor trophy indicators, due to their expected wide ecological amplitude. Finally, the response of species complexes and their members (cryptic species) to a trophic gradient (phosphorus) were compared. Multivariate analyses showed similar efficiency of all three taxonomic resolutions in depicting community patterns and their environmental correlates, suggesting that even genus level resolution is sufficient for routine bioassessment of shallow fishponds with a wide trophic range. However, after controlling for coarse taxonomic matrices, fine taxonomic resolution (with resolved cryptic species) still showed sufficient variance related to the environmental variable (habitat groups), and increased the sharpness of classification, number of indicator species for habitat categories, and gave better separation of habitat categories in the ordination space. Regression analysis of trophic bioindication and phosphorus concentration showed a weak relationship for species complexes but a close relationship for the remaining taxa. GLM models also showed that no species complex responded to phosphorus concentration. It follows that the studied species complexes have wide tolerances to, and no apparent optima for, phosphorus concentrations. In contrast, various responses (linear, unimodal, or no response) of cryptic species within species complexes were found to total phosphorus concentration. In some cases, fine taxonomic resolution to species level including cryptic species has the potential to improve data interpretation and extrapolation, supporting recent views of species surrogacy.

Keywords: epiphyton, cryptic species, ecological resolution, taxonomic resolution, indicator species

5. CONCLUSIONS

Diatoms form complex and structured films on plant substrate. Significant differences were found in species richness of different ecoregions and water types. Altogether, 131 taxa were found in 25 fishponds and 13 small streams located in the Svitava River Basin and the White Carpathian Mountains in the Czech Republic (Kollár et al., 2015). In the meso-oligotrophic alpine Lake Valagola 78 taxa were identified (Letáková et al., 2017). And finally 263 taxa were found in the study focused on ponds of various characteristics appearing all over the Czech Republic (Poulíčková et al., 2016), where species diversity varied from 11 to 51 taxa. The most species-rich place was the dystrophic Novozámecký Pond, the smallest taxon amount was found in the highly eutrophied Staňkovský Pond. The dominant epiphytic diatom *Achnanthidium minutissimum* agg. (Kollár et al., 2015; Poulíčková et al., 2017) was able to create even 88% of the community (Kollár et al., 2015). The other two most frequently occurring species (Kollár et al., 2015; Poulíčková et al., 2017) were *Gomphonema parvulum* agg. with representation 1–48% and *Cocconeis placentula* Ehrenberg with representation 1–100% (Kollár et al., 2015). Species complexes represented the majority of epiphytic assemblages but this dominance did not lead automatically to a reduction of overall diversity of the sample. *Fragilaria polonica* M. Witak & Lange-Bertalot and *Staurosirella pinnata* (Ehrenberg) D. M. Williams & Round were the most common species in Lake Valagola (Letáková et al., 2016).

CCA analysis showed significant influence of pH and water streaming – running/stagnant water (Kollár et al., 2015). Species correlating with higher pH were *Amphora pediculus* (Kützing) Grunow, *Cocconeis pediculus* Ehrenberg, *Cymbella excisiformis* Krammer, *Encyonopsis cesatii* (Rabenhorst) Krammer, *Encyonopsis microcephala* (Grunow) Krammer, *Eunotia arcus* Ehrenberg, *Gomphonema pumilum* (Grunow) E. Reichardt & Lange-Bertalot, *Nitzschia palaeformis* Hustedt and *Nitzschia* *sinuata* (Thwaites) Grunow, lower pH was preferred by *Mayamaea atomus* (Kützing) Lange-Bertalot, *Planothidium ellipticum* (Cleve) M. B. Edlund, *Planothidium lanceolatum* (Brébisson ex Kützing) Lange-Bertalot and *Nitzschia palea* (Kützing) W.Smith. Typical for lentic waters were diatoms like *Fragilaria brevistriata (recently accepted name Pseudostaurosira brevistriata* (Grunow) D.M. Williams & Round), *Encyonopsis microcephala* (Grunow) Krammer, *Eunotia arcus* Ehrenberg, *Denticula tenuis* Kützing, lotic waters often contained *Cocconeis pediculus* Ehrenberg, *Gomphonema angustatum* (Kützing) Rabenhorst and *Navicula tripunctata* (O. F. Müller) Bory.

Regression analysis confirmed that epiphytic diatom assemblages reflect trophic gradient, although there are significant differences in the relationship of species complexes and remaining taxa to phosphorus concentrations (Poulíčková et al., 2017). Oligo or mesotrophic waters were preferred by *Achnanthidium minutissiumum* sensu stricto (Kützing) Czarnecki, *Achnanthidium lineare* W. Smith, *Achnanthidium caledonicum (Lange-Bertalot) Lange-Bertalot*, *Gomphonema exilissimum* (Grunow) Lange-Bertalot & Reichardt. Eutro or hypertrophic waters were correlated with *Achnanthidium eutrophilum* (Lange-Bertalot) Lange-Bertalot, *Achnanthidium straubianum* (Lange-Bertalot) Lange-Bertalot, *Gomphonema parvulum* (Kützing) Kützing.

The spatial distribution of epiphytic diatoms is also influenced by host plant distribution (Letáková et al., 2016).

The influence of the substrate on the composition of the epiphytic community did not show uniform results in the localities investigated. No significant specificity was noted in the Czech ponds and small streams except *Lemna minor* L. Species such as *Fragilaria brevistriata (Pseudostaurosira brevistriata)*, *Staurosirella pinnata* or *Nitzschia palaeformis* avoid *Lemna minor* as a host plant. *Lemnicola hungarica* (Grunow) Round & Basson, a diatom typical for *Lemna minor*, inhabited also *Phragmites australis* (Cav.) Steud in the

Svitava river basin (Kollár et al., 2015). In the case of the oligo-mesotrophic Lake Valagola, significant differences were found in diatom species richness, diversity and composition between the two main host plants. *Potamogeton gramineus* L. assemblages were characterized by higher species richness and diversity, and by the large-celled, adnate diatom species *Epithemia adnata* (Kützing) Brébisson, *Rhopalodia gibba* (Ehrenberg) Otto Müller, *Eunotia arcus* Ehrenberg and *E. arcubus* Nörpel & Lange-Bertalot. *Chara aspera* Willd. was preferred by the small-celled, motile diatom species *Brachysira neoexilis* Lange-Bertalot and *Encyonopsis cesatii* (Rabenhorst) Krammer (Letáková et al., 2016).

It has been proved then, that epiphytic diatoms are suitable for bioindication in generally more nutrient rich Czech fishponds and rivers, as their distribution is not biased by plant substrate type. However, *Lemna* sp. should be excluded from the macrophytes used for this purpose.

Surprisingly, species complexes represented the majority of epiphytic assemblages (up to 97%) with no significant differences between lotic and lentic waters or substrates. In the case of pond epiphyton (Poulíčková et al., 2017), 18 species complexes were formed by 43 cryptic species and created 3-97% of the community (average 56,81 ± standard deviation 25,19 %). The most common was the complex of *Achnanthidium minutissimum* agg. (with the following morphotypes: *Achnanthidium affine* (Grunow) Czarnecki, *Achnanthidium caledonicum* (Lange-Bertalot) Lange-Bertalot, *Achnanthidium eutrophilum* (Lange-Bertalot) Lange-Bertalot, *Achnanthidium jackii* Rabenhorst, *Achnanthidium lineare* W. Smith, *Achnanthidium minutissimum* (Kützing) Czarnecki and *Achnanthidium straubianum* (Lange-Bertalot) Lange-Bertalot. Even though the research in Valagola was focused in a different way, out of these 18 species complexes, *Achnanthidium minutissimum, Amphora pediculus, Cocconeis placentula* and *Gomphonema parvulum* species complexes have been noticed.

Precise taxonomical identification did not play such an important role in biomonitoring as could have been expected. Simple genus level identification of epiphytic diatoms brought sufficient information required for normal routine biomonitoring (Poulíčková et al., 2017). However, fine taxonomic resolution (with resolved cryptic species) still showed sufficient variance related to the environmental variable (habitat groups), and increased the sharpness of classification, number of indicator species for habitat categories, and gave better separation of habitat categories in the ordination space. Species complexes showed low sensitivity to changes in phosphorus concentration, and thus seems to have low indicatory power in trophic state bioassassment (Poulíčková et al., 2017).

Ecological heterogeneity exists within species complexes. Species complexes of epiphytic diatoms might be divided into three groups: 1. cryptic species with indistinguishable frustule morphology, lacking keys for identification, and/or with limited information on their distribution and ecology (e.g. *Navicula cryptocephala* agg.). These are worthy of future attention and investigation to fill in the information gap, and to allow them to be transferred into one of the following groups: 2. cryptic species with low potential for improving indicative power due to shared ecology, or without a response to the gradient studied (e.g. *Fragilaria capucina* agg., *Encyonema/Cymbella* agg.); 3. morphologically (LM) more or less distinguishable cryptic species that are ecologically differentiated (*Achnanthidium minutissimum* agg., *Gomphonema parvulum* agg., *Nitzschia palea* agg.), with high potential for use in biomonitoring (Poulíčková et al, 2017).

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I. Letáková M., Fránková M. & Poulíčková A. (2018): Ecology and applications of freshwater epiphytic diatoms – review. *Cryptogamie, Algologie* 39(1):1-20.

II. Fránková M., Poulíčková A., Tihlaříková E., Neděla V., Šumberová K. & Letáková M. (submitted): The low temperature method for Environmental Scanning Electron Microscopy – a new tool for observation of diatom assemblages *in vivo*. *Diatom Research*.

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V. Poulíčková A., Letáková M., Hašler P., Cox E. & Duchoslav M. (2017): Species complexes within epiphytic diatoms and their relevance for the bioindication of trophic status. *Science of the Total Environment* 599-600: 820-833.

8. PRESENTATIONS AT MEETINGS

- LETÁKOVÁ M., CANTONATI M., HAŠLER P., ANGELI N., & POULÍČKOVÁ A. (2015): Substrate specificity and fine-scale distribution of epiphytic diatoms in a shallow tarn in the Brenta Dolomites (South-eastern Alps). 15th -19th June, 2015, 9th Use of Algae for Monitoring Rivers and comparable habitats (UAMRIch) and International Workshop on Benthic Algae Taxonomy (Inbat). (poster). – *awarded by the price for the best student poster presentation*
- LETÁKOVÁ M., HAŠLER P., DUCHOSLAV M. & POULÍČKOVÁ A. (2016): Epiphytic diatoms in the ponds of the Czech Republic. 19th-21st September 2016, 57th Meeting of the Czech Phycological Society, Prague. (poster)

9. PARTICIPATION ON PROJECTS

- Participation on a project focused on monitoring of the Czech wetlands (the Ramsar Convention) coordinated by Ministry of the Environment of the Czech Republic.
- Internal grant agency of Palacký University PrF-2014-001, PrF-2015-001, PrF-2016-001 and PrF-2017-001.

10. SOUHRN: (SUMMARY, IN CZECH)

Tato disertační práce je zaměřena na jednu skupinu bentických rozsivek – na epifytické rozsivky, tj. na ty, které žijí přisedle na rostlinném substrátu. Mnoho aspektů jejich života není totiž stále dostatečně objasněno, přestože mohou tvořit velmi podstatnou část primární produkce a navzdory jejich důležitosti v biomonitoringu a paleolimnologických rekonstrukcích. Byla zkoumána substrátová specifita, diverzita epifytických rozsivek, rozsah druhových komplexů ve společenstvu epifytických rozsivek a také metody používané pro hodnocení ekologického stavu vod.

Tato studie epifytických rozsivek byla založena na vzorkování, měření ekologických parametrů v terénu, na přípravě trvalých rozsivkových preparátů, analýze druhového složení rozsivkových společenstev a na statistickém zpracování získaných dat. Cílem práce bylo najít odpovědi na následující otázky: i) Reflektuje složení epifytických rozsivek ekologické parametry lokality? ii) Ovlivňuje typ vodní rostliny složení společenstva epifytických rozsivek? iii) Jaká je celková diverzita společenstva epifytických rozsivek? iv) Jak velká část společenstva je tvořena problematickými druhovými komplexy, a jaké je jejich složení? v) Reflektují nerozlišené druhové komplexy stav vody méně přesně než jiné druhy? vi) Existují druhové komplexy, jejichž rozlišení by mohlo zkvalitnit biomonitoring?

Epifytické rozsivky odpovídaly na ekologické parametry vody ve všech zkoumaných lokalitách. Substrátová specifita byla zanedbatelná ve vodách vyšší trofie, v čistém horském jezeře Valagola byla nalezena signifikantní substrátová specifita. Skupina epifytických rozsivek má celkově velkou druhovou bohatost a podstatná část je tvořena druhovými komplexy, tvořenými kryptickými a semikryptickými druhy. Ačkoliv se pouhé rodové určení zdá být naprosto dostačující pro rutinní monitoring, jemné taxonomické rozlišení stále vykazuje odchylku v souvislosti s proměnnými prostředí a zvyšuje přesnost klasifikace.

Nerozlišené druhové komplexy měly nízkou citlivost pro změny v koncentraci fosforu. Některé kryptické druhy mají potenciál zlepšit bioindikační modely. Proto rozlišení těchto druhových komplexů je zásadní a naléhavou záležitostí, která by vedla mimo jiné k lepšímu pochopení ekologického stavu vod.