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Pond turtle carapaces, an alternative natural substrate for the use of a diatom-based water quality index

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ABSTRACT

Benthic diatoms are widely used as bio-indicators in water quality monitoring of rivers and streams, but less commonly of stagnant waterbodies such as pools and lakes. Currently, a new method for assessing the ecological status of lakes is being tested in France to meet the requirements of the European Water Framework Directive (WFD) 2000/60/EC. This new method uses epilithic and epiphytic diatoms growing on stones and aquatic plants. Alternatively, freshwater turtles and the epibiotic algae found on their carapaces may provide an excellent substrate for estimating water quality. Our study proposes an original diatom-based method for assessing the quality of water bodies in which turtles live. The main objective was to compare the Biological Diatom Index (BDI) values measured from epizoic diatom samples taken on the carapace of turtles with classical epilithic or epiphytic samples. Epizoic samples were taken from the carapaces of the turtle *Emys orbicularis* in two shallow French Mediterranean wetlands. Our results indicate that diatom assemblages differ slightly within the three substrates studied with limited variations in index value. However, some variability among results from the same site suggests that several turtle individuals should be sampled.

KEYWORDS: Epizoic diatoms; *Emys orbicularis*; epilithic diatoms; epiphytic diatoms; wetland; biomonitoring; BDI; water quality assessment

Introduction

Primary producers (phytoplankton, phytobenthos and macrophytes) are widely used as ecological indicators in the European Water Framework Directive (WFD) 2000/60/EC (European Union 2000, Kelly 2013). Diatoms (Bacillariophyceae), small unicellular algae characterized by the presence of an outer silica carapace (Round et al. 1990), are considered one of the most powerful bio-indicators due to their short generation times, their high diversity and their fast reaction to even very slight variations in the physico-chemical characteristics of their aquatic environment (pH, salinity, organic matter, nutrients, metal pollution). Diatoms are commonly found on all kinds of submerged vegetal and inert mineral substrates such as the submerged parts of helophytes, aquatic plants, stones and sand grains (Kelly et al. 1998). The presence of a broad array of taxa indicating low water quality provides an excellent early warning signal of degraded water conditions for managers (Kelly et al. 2016).

In France, the Biological Diatom Index (BDI) has been commonly used in the past 15 years for monitoring running waters (AFNOR 2007, 2016; Coste et al. 2009). The index reflects the level of organic (saprobity) and trophic (nitrogen) pollution, as well as contamination by toxic substances such as mineral or synthetic micropollutants. Currently, a diatom-based method for assessing the ecological status of European lakes is being tested in France to meet the European Water Framework Directive requirements (WFD, 2000/60/EC) for stagnant waterbodies. The sampling method uses both epilithic and epiphytic diatoms. Apart from living on these inert substrates, diatoms found epizoically on many aquatic animals (e.g. Wu & Bergey 2017, Ferrario

et al. 2018), could likewise provide an excellent population for the application of water quality indices.

Freshwater turtles such as the European pond turtle (*Emys orbicularis* Linnaeus, 1758) are an important component of freshwater ecosystems worldwide. This species has a vast longitudinal and latitudinal range of distribution, from Portugal to the Aral Sea and from North Africa to Russia, respectively (Fritz 2003). Their large carapaces provide a perfect inert natural and challenging habitat for the growth of epizoic algae. In the past, a fairly large diversity of different algal groups has been described on the carapaces and skin of freshwater turtles (Edgren et al. 1953; Soylu et al. 2006; Garbary et al. 2007; Wetzel et al. 2010, 2012; Wu & Bergey 2017). A study on the European pond turtle in the Camargue region (South France) identified 77 epizoic algal taxa on the carapaces, amongst which diatoms formed the most diverse group with 51 recorded taxa (Fayolle et al. 2016).

We hypothesize in the present study that epizoic diatoms living on turtle carapaces can be used as water quality indicators. In the present study we investigated in two different Mediterranean localities, (1) whether diatom assemblages on *Emys orbicularis* were habitat-specific or similar to those living on the surrounding mineral or vegetal substrates, and (2) the consequences on the BDI of the choice of an epizoic instead of an epilithic or epiphytic diatom sample. The final goal is to extend the possibilities of diatom sampling for the application of a diatom index, especially in sites where mineral or vegetal substrates are not available.

Materials and methods

The two sampling localities investigated in this study, are located in the south of France. The first site (Charpines, 43°71'48" N, 5°31'42" E, altitude 147 m, date of sampling:

06/29/2016) is a wetland with a small surface area (0.22 ha), shallow and very silted (maximum water depth of 1 m and 0.10 m on accessible shores), located near the lower reaches of the Durance River. Following the collapse of the riverbed (Renet et al. 2017), this wetland is only connected to the Durance during major floods. The inflow comes from a water treatment plant resulting in an overall low water quality. The second site (Esquineau, 43°51'20" N, 4°66'76" E, altitude 1 m, sampling date: 06/08/2017) is a vast (16 ha) rather shallow (average water depth of 50 cm) wetland area located in the natural reserve of the Tour du Valat in the Camargue region and composed of semi-permanent marshes and man-made managed irrigation canals (Olivier et al. 2010). The wetland area has a moderate water quality.

Epilithic and epiphytic diatoms were sampled as recommended by the IRSTEA method (DCE Plan d'eau Phytobenthos Irstea REBX Version 1.2 February 2013). The epilithic and epiphytic samples were collected separately as pooled biofilm extracts from at least five pebbles which had been previously placed in both sites for 4 weeks, and five submerged helophyte stems (*Phragmites australis* (Cav.) Trin. ex Steud.), respectively.

The two sites host two distinct turtle populations that are yearly monitored using capture-recapture methods (Ficheux et al. 2014; Renet et al. 2017). Epizoic diatoms were collected at each site by brushing off an area of 20 cm² on the back of ten adult turtle carapaces using single-use toothbrushes. Half of the samples were pooled to form a single mixed epizoic sample. The resulting number of samples from each site was 13 (11 epizoic, one epilithic and one epiphytic). The samples were preserved in 90 % ethanol.

Diatom identifications were performed after having prepared permanent slides following BDI standard NF T90-354. The organic matter in the small fraction of raw

samples was digested with hot (90°C., ±10h) concentrated solution of hydrogen peroxide (30% v/v) using a digestion block system (DigiPREP systems, SCP Science) and calcium salts were dissolved with concentrated hydrochloric acid (30% v/v). Following digestion and decantation, cleaned material was rinsed and diluted with deionized water and mounted permanently on glass slides using Naphrax® resin (refractive index = 1.74). Light microscopy (LM) analysis was performed using a Nikon (Eclipse N*i*) light microscope equipped with phase contrast plan apochromat ×100 oil optic. In each sample, at least 400 valves were identified to the lowest taxonomic level possible and counted along random transects. Most taxa were determined according to Lange-Bertalot (2001, 2017), Krammer (2002, 2003), Levkov et al. (2016) and Lange-Bertalot et al. (2017). The BDI was calculated from diatom counts using Omnidia software, version 6.0.8 (2018) (Lecointe et al. 1993). Data analysis was performed using R (R Core Team 2018). The comparison of epizoic, epiphytic and epilithic diatom communities was carried out on the basis of their specific composition using Ward's hierarchical classification method with Canberra distance. Only the relative abundances of species with at least three valves counted were considered. The IndVal analysis of indicator species in clusters was done using the labdsv R package (Roberts 2019). BDI values between clusters were compared using the non-parametric Kruskal-Wallis test.

Results

A total of 81 diatoms taxa (including species, varieties and forms, with abundance ≥ 2 valves) were identified in 26 samples: 28 were recorded in Charpines and 53 in Esquineau (Supplementary material 1). At both sites, the cluster analysis did not show a strong differentiation between communities taken from conventional substrates and from turtle carapaces (Fig. 1). Indeed, the first node of each tree separated epizoic

samples, which means that the type of substrate was not the most important factor determining diatom assemblages. The analysis also revealed considerable heterogeneity among epizoic communities. However, the pattern was not the same at the two sites. In Charpines, three main groups could be identified: the first one included only the epiphytic and epilithic samples (n=2). This first group was closer to most epizoic samples, forming Group 2 (n=7), than to a third marginal group of 4 epizoic samples (Fig 1a). In Esquineau, the communities were more homogeneous than in Charpines, but similarly, three groups could likewise be distinguished (Fig. 1b). In a first large cluster, the epilithic and epiphytic as well as most epizoic samples were grouped (n=9). Two pairs of samples, forming groups 2 and 3, were separated from the main group by an increasing distance, corresponding to an increasingly different composition of communities.

The consequences of variations in communities between groups of substrates on the diatomic index BDI was greater in Charpines than in Esquineau. Indeed, group 3 of epizoic samples from Charpines had significantly higher BDI ($p<0.05$, Kruskall-Wallis test), between 3 and 4 points above the values of the other groups (Fig. 1a). These differences in BDI could lead to different assessments of water quality (poor or bad) depending on the samples considered for this site. The communities in Group 3 were characterized by *Sellaphora pupula* and *Nitzschia linearis*, both species showing relatively high specific BDI values (Table 1). In Esquineau, the differences in BDI between the three groups were smaller (between 1 and 2 points, $p=0.05$, Fig 1b). Groups 2 and 3 had lower values and were characterized by indicator species such as *Tryblionella littoralis* or *Craticula cuspidata* (group 2) and *Fragilaria gracilis* or *Mastogloia smithii* (group 3, Table 1). Nevertheless, considering only the mixed epizoic samples being representative of the carapace substrate, the absolute differences in BDI

between epizoic and standard samples were similar in Charpines and Esquineau (≤1.5, Figure 1).

Discussion

The main result of this study was a higher degree of similarity in diatom communities and BDI values between samples from *Emys orbicularis* carapaces and from stones or macrophytes than we expected, given the specific characteristics of the animal substrate. The chitin structure of the carapaces and the production of secretions and excreta by turtles (Edgren et al. 1953) could be selection factors for epizoic species. The movements of the turtles may also increase the risk of species abrasion, especially in case of friction between the carapace and the macrophyte vegetation in which the turtles live. It also generates variable abiotic conditions that can be stressful for microalgae. Light intensity on the carapace may change considerably according to the position of the turtle in the water column. Moreover, European pond turtles often exit their aquatic environment for basking at sunlight. This out-of-water thermoregulation activity can last several hours in spring when the water and air temperatures are low. The resulting desiccation of the biofilm on the carapace during sun exposure may probably affect the composition of attached diatom communities. Recurrent emersion may be a strong pressure that epilithic or epiphytic biofilm may not commonly experience, unless there are large water level fluctuations. Also, the overall similarity of the communities on stones, macrophytes and turtles carapace suggests that the type of substrate is not a determinant of periphyton specific composition as strong as the water quality (Cellamare et al. 2012) or the date of sampling (Cetin 2008). It can also be assumed a high turnover of periphyton on turtle carapaces due to frequent attachment/detachment in relation to host motion and therefore little time for selection of most adapted species

to operate. This 'mass effect' hypothesis (Shmida and Wilson 1985) could explain why there is no clear differentiation between epizoic and epiphytic or epilithic communities.

However, some differences were observed between turtles especially in the Charpines site. They may be due to differences between individuals in size, age, sex or behaviour. If the turtles can be considered, because of their movement, more representative of a water body than stones or macrophytes, they may use only a part of a lake and even expand their living area to another neighboring freshwater system (Olivier 2002) . The case of the Charpines site showed that there is a risk of considerable deviation of the BDI and misclassification if only one individual is considered. Thus, it should be recommended to sample biofilm on several individuals to reduce variance, analogously as for stones in the BDI protocol. In addition, it may be preferable to select individuals of the same species to avoid more variability due to differences in the ecology of chelonian species (Soylu et al. 2006), such as *Emys orbicularis* and *Mauremys leprosa* in Europe. The BDI, initially developed for running waters, may be replaced in the next years by a new index more appropriate for lakes or wetlands. However, the conclusion of this study concerning the suitability of the turtle carapace substrate for diatom bioindication should be still valid when another specific index will be used.

Conclusion

The method applied in this study represents a potential tool for the assessment of wetlands where *Emys orbicularis* is monitored using CMR methods. It implies the application of a diatom index such as the BDI, on epizoic diatoms. The European pond turtle carapace offers an alternative substrate in wetlands without stone or macrophytes available for sampling, allowing managers to monitor in the same time a protected species and the ecological status of wetlands which are generally not included in the

national WFD networks.

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Luc Ector is a botanist and senior researcher at the Luxembourg Institute of Science and Technology. He has written over 210 articles and has been working on diatoms in rivers, lakes and soils for the last 30 years. He was the President of the "Association des Diatomistes de Langue Française (ADLaF)", which organizes annual meetings on

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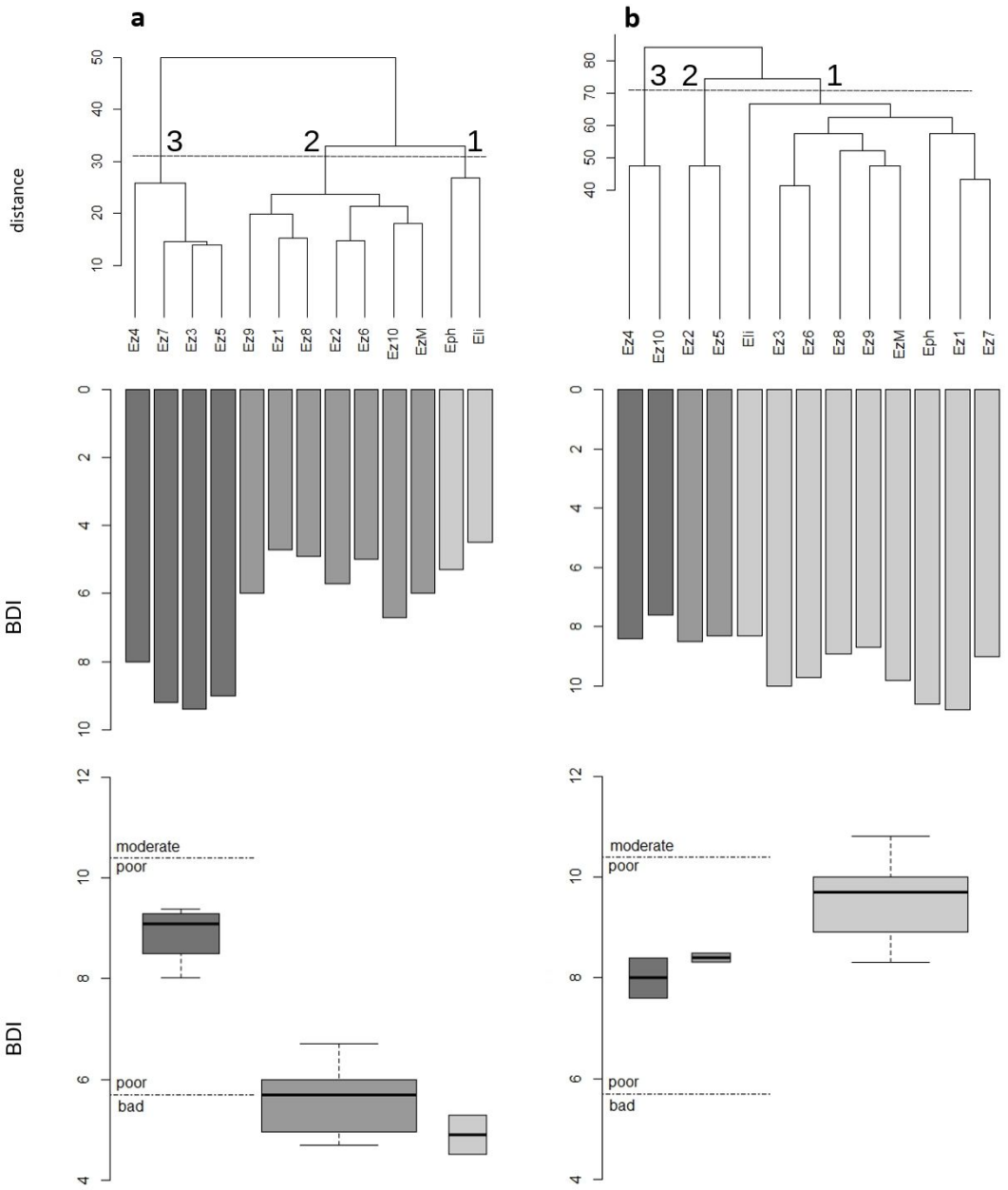
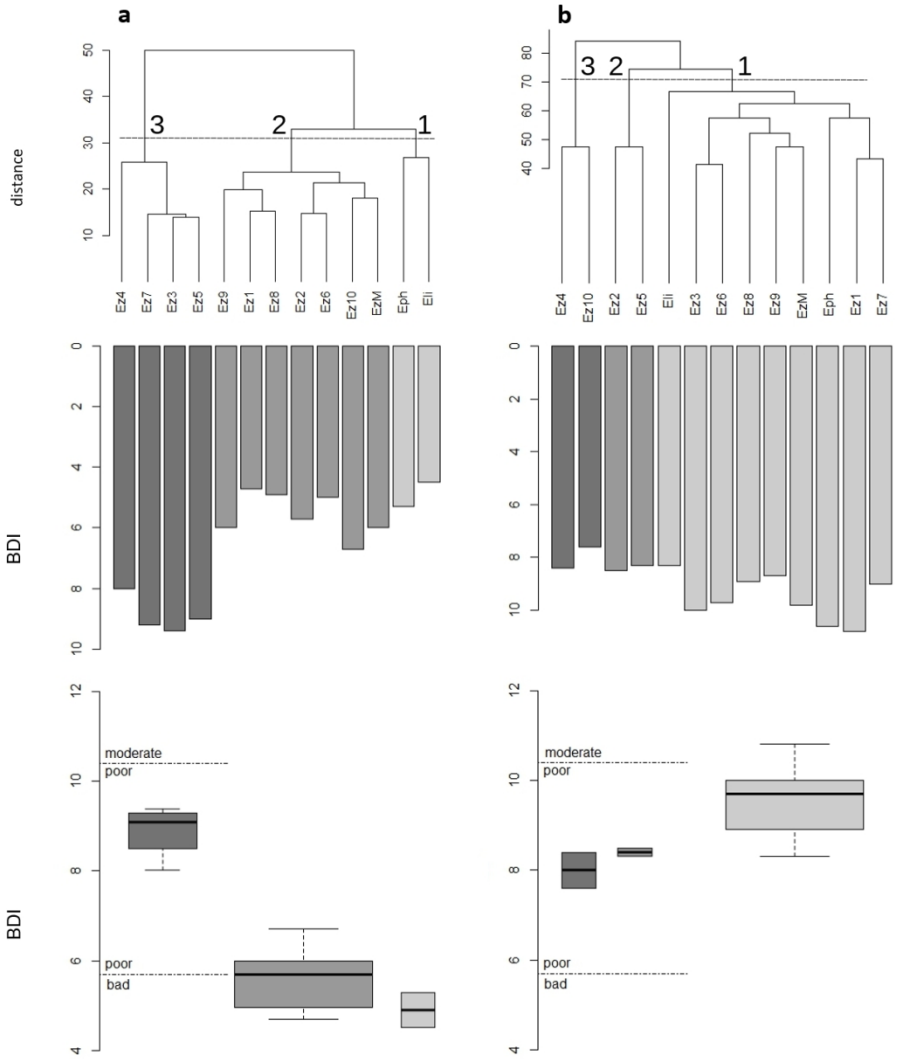
Table 1. Indicator species of the three clusters considered in each site with indication of their IndVal value and their BDI score (0 to 20).

Site	Diatom species	cluster	Ind-Val	p-val	BDI score
Charpines					
	Eolimna subminuscula (Manguin) Gerd Moser, Lange-Bertalot & Metzeltin	1	0.83	0.003	4.3
	Nitzschia sp Hassall	1	0.80	0.025	nd
	Planothidium frequentissimum (Lange-Bertalot) Lange–Bertalot	1	0.92	0.025	11.7
	Cocconeis placentula Ehrenberg	2	0.84	0.017	15.4
	Diadesmis confervacea Kützing	2	0.68	0.006	1
	Luticola goeppertiana (Bleisch) D.G.Mann in Round et al.	2	0.73	0.001	5
	Nitzschia amphibia Grunow	2	0,75	0.001	7.7
	Nitzschia palea (Kützing) W.Smith	2	0.65	0.002	5
	Pseudostaurosira trainori E.Morales	2	0.83	0.002	nd
	Sellaphora seminulum (Grunow) D.G.Mann	2	0.67	0.001	2.9
	Lemnicola hungarica (Grunow) Round & Basson	3	0.94	0.001	3
	Nitzschia linearis (C.Agardh) W.Smith	3	0.90	0.003	12.1
	Sellaphora pupula (Kützing) Mereschkowski	3	0.85	0.005	9.4
	Tryblionella littoralis (Grunow) D.G.Mann in Round et al.	3	0.75	0.049	2.9
Esquineau					
	Eunotia bilunaris (Ehrenberg) Schaarschmidt	1	0.63	0.029	20
	Navicula cryptotenella Lange-Bertalot	1	0.73	0.003	16
	Navicula lundii E.Reichardt	1	0.84	0.003	12.7
	Sellaphora seminulum (Grunow) D.G.Mann	1	0.81	0.002	2.9
	Amphora copulata (Kützing) Schoeman & R.E.M.Archibald	2	0.82	0.007	14.2
	Craticula cuspidata (Kützing) D.G.Mann in Round et al.	2	1.00	0.017	2.6
	Pinnularia sp. Ehrenberg	2	0.88	0.011	nd
	Sellaphora pupula (Kützing) Mereschkowski	2	0.68	0.017	9.4
	Tryblionella littoralis (Grunow) D.G.Mann in Round et al.	2	0.93	0.045	2.9
	Diploneis Ehrenberg ex Cleve	3	0.96	0.028	nd
	Fragilaria gracilis Østrup	3	0.90	0.035	17
	Mastogloia smithii Thwaites	3	1.00	0.028	nd
	Navicula simulata Manguin	3	0.83	0.022	9.2
	Navicula veneta Kützing	3	0.59	0.005	2.3
	Tabularia fasciculata (C.Agardh) D.M.Williams & Round	3	0.60	0.041	3.5
	nd = no data				

Figure 1. Cluster analysis and BDI values of the diatom samples collected at Charpines (a) and Esquineau (b) sites. Three clusters (numbered 1 to 3) have been considered in each site. BDI values were represented for each sample separately (middle barplots) and in each cluster (bottom boxplots). Eph= epiphytic, Eli=epilithic, Ez1-10= epizoic, EzM= mixed epizoic sample.





228x271mm (150 x 150 DPI)

Supplementary material 1. Percentage abundance of the diatom species identified in the Charpines and Esquineau sites. EzM= mixed epizoic samples, Eph= epiphytic sample, Eli= epilithic sample.

Diatom species	Charpines			Esquineau		
% (occurrence ≥2 valves)	EzM	Eph	Eli	EzM	Eph	Eli
Amphora copulata (Kützing) Schoeman & R.E.M.Archibald				2		1
Achnanthidium minutissimum (Kützing) Czarnecki	2.7	1.2		0.8	1.2	
Amphora pediculus (Kützing) Grunow					0.5	
Anomoeoneis sphaerophora (Ehrenberg) Pfitzer	0.5					0.5
Bacillaria paxillifera (O.F.Müller) T.Marsson				1.3		
Cocconeis placentula Ehrenberg	0.5					
Craticula buderi (Hustedt) Lange-Bertalot	0.7			0.5	1	
Diadesmis confervacea Kützing	0.5					
Encyonema ventricosum (Kützing) Grunow in Schmidt et al.			0.5			
Eolimna minima (Grunow) Lange-Bertalot	2.7		4.3	1	1.7	2
Eolimna subminuscula (Manguin) Gerd Moser, Lange-Bertalot & Metzeltin	1.7	2.5	24			
Eunotia bilunaris (Ehrenberg) Schaarschmidt				5.8	7.5	1
Eunotia soleirolii (Kützing) Rabenhorst				1.8		
Eunotia Ehrenberg				1.3		
Fallacia pygmaea ssp. *subpygmaea* Lange-Bertalot, Cavacini, Tagliaventi & Alfinito	0.7					
Fistulifera saprophila (Lange-Bertalot & Bonik) Lange-Bertalot			1.3			
Fragilaria famelica (Kützing) Lange-Bertalot					3	
Fragilaria gracilis Østrup				2.3	1	
Geissleria acceptata (Hustedt) Lange-Bertalot & Metzeltin				0.5		0.5
Gomphonema acidoclinatum Lange-Bertalot & E.Reichardt					1.2	0.7
Gomphonema affine var. *rhombicum* E.Reichardt				1.8	1	
Gomphonema capitatum Ehrenberg					6	
Gomphonema clavatum Ehrenberg				1	6.2	1
Gomphonema gracile Ehrenberg					1.2	1
Gomphonema parvulum Kützing	1.7	74.1	3.8	0.5	2.2	
Gomphonema saprophilum (Lange-Bertalot & E.Reichardt) N.Abarca, R.Jahn, J.Zimmermann & Enke	11.9	5.7	2.3	0.8	1.5	
Luticola goeppertiana (Bleisch) D.G.Mann in Round et al.	9	1.2				
Mayamaea Lange-Bertalot			0.8	1		
Mayamaea permitis (Hustedt) Bruder & Medlin			1	0.5		1
Navicula antonii Lange-Bertalot						4.5
Navicula cincta (Ehrenberg) Ralfs in Pritchard					0.7	
Navicula cryptotenella Lange-Bertalot				2	2	1
Navicula eidrigiana J.R.Carter				1.3	1.5	
Navicula erifuga Lange-Bertalot in Krammer & Lange-Bertalot				2	4.7	0.5
Navicula gregaria Donkin				1.3		
Navicula lundii E.Reichardt				3.3	6	1.2

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Navicula simulata Manguin				0.5		
Navicula trivialis Lange-Bertalot	0.5			7.8		
Navicula veneta Kützing	3.7		3.8	12.3	22.2	8.7
Naviculadicta cosmopolitana Lange-Bertalot				0.5		
Nitzschia Hassall		1.2	0.5			
Nitzschia amphibia Grunow	5.2	1.2	0.5		4.7	1.5
Nitzschia dissipata (Kützing) Grunow				0.5		
Nitzschia filiformis var. *conferta* (P.G.Richter) Lange-Bertalot				3.8		
Nitzschia frustulum (Kützing) Grunow	4.5	3.5	33			
Nitzschia frustulum var. *subsalina* Hustedt					1.2	
Nitzschia inconspicua Grunow				4.5	3.5	23.9
Nitzschia liebethruthii Rabenhorst				1.3	0.5	
Nitzschia linearis (C.Agardh) W.Smith	8.2				0.5	
Nitzschia linearis var. *tenuis* (W.Smith) Grunow in Cleve & Grunow				0.5		
Nitzschia microcephala Grunow in Cleve & Möller				1.8		
Nitzschia palea (Kützing) W.Smith	15.2	4	11	11.3	4.2	29.2
Nitzschia palea var. *debilis* (Kützing) Grunow in Cleve & Grunow	3.5			3.8		7
Nitzschia solita Hustedt				1.5		
Pinnularia lata (Brébisson) Rabenhorst				0.5		
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot			1.5	1		
Pseudostaurosira trainori E.Morales	0.7					
Sellaphora pupula (Kützing) Mereschkowksy	10.9	1.7	2	1.3		0.7
Sellaphora seminulum (Grunow) D.G.Mann	12.7	0.5	8	6.8	4.5	11.5
Stauroneis kriegeri R.M.Patrick		0.5				
Tabularia fasciculata (C.Agardh) D.M.Williams & Round				1.5	4.7	
Tryblionella hungarica (Grunow) D.G.Mann in Round et al.				3.5		
Ulnaria acus (Kützing) Aboal in Aboal et al.					1.2	
Ulnaria biceps (Kützing) Compère				0.8		

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138x68mm (96 x 96 DPI)