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Diatom Indicators of Fluctuating/Intermittent Discharge from Springs in Two Bavarian Nature Conservation Areas

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Citation: Cantonati, M.; Casoria, C.; Gerecke, R.; Bilous, O.P.; Maisto, G.; Segadelli, S.; Spitale, D.; Steinbauer, A.; Vogel, S.; Saber, A.A. Diatom Indicators of Fluctuating/ Intermittent Discharge from Springs in Two Bavarian Nature Conservation Areas. *Diversity* **2023**, *15*, 915. https://doi.org/10.3390/d15080915

Academic Editors: Davide Bassi and Michael Wink

Received: 22 June 2023
Revised: 2 August 2023
Accepted: 3 August 2023
Published: 7 August 2023

Abstract: As a follow-up to the project "Springs in the Bavarian National Parks as Indicators of Climate Change (SpringNPB)", a standard methodology for using springs as sentinel environments of climate change was transferred to the UNESCO Rhön Biosphere Reserve and other Bavarian middle-elevation mountain ranges. We studied diatoms from fifteen springs selected in the UNESCO Biosphere Reserve (9) and Steigerwald Nature Park (6). A total of 127 species belonging to 40 genera were found sampling 3 microhabitat types (lithic materials, hygrophilous or aquatic vegetation, and surface sediments). The cumulative percentage of endangered species according to the Red List was 41.5%. These very shaded, low–medium conductivity, low-discharge forest springs are fed by small surficial aquifers. As a consequence, the discharge fluctuates widely, and some springs even occasionally fall dry. Our results could contribute to the use of diatoms as indicators of discharge variability/desiccation in springs: springs affected by discharge variability have lower diatom species richness and distinct diatom communities; diatom indicators and metrics can be validated using invertebrates; larger databases will be necessary to identify the most suitable diatom indicators.

Keywords: diatoms; springs; ecological characteristics; climate change effects; discharge variability; Rhön Biosphere Reserve; Steigerwald Nature Park

1. Introduction

A spring can be defined as the point where an aquifer meets the Earth's surface or where the groundwater emerges on the surface through fractures, faults in the rock, or depressions. Springs represent ecosystems dependent on groundwater (GDEs): Emerging on the surface when they intersect the Earth's surface, they have physical and chemical properties dependent on the aquifer generating them [1]. Springs are frequent throughout landscapes, but the morphology and the dimensions vary considerably: from slight infiltrations of rocky walls to alluvial springs of river landscapes, from karst resurgences to openings that emerge, discharging many thousands of liters of water per second [2].

The number of sources is unknown for many geographic regions. Wanting to extrapolate from the estimates on the mainland, excluding Antarctica, there would be 4 springs per 10 km^2 and 7 hot springs per 10,000 km^2. These numbers are underestimated because they do not take into account the much more numerous small sources [3].

The study of springs is called crenobiology, a special field of limnology, to emphasize the presence of unusual characteristics and a special biota. The term was coined about sixty years ago by Illies and Botosaneanu [4].

Springs are widely recognized as important habitats and biodiversity hotspots [5]. These are unique aquatic habitats that contribute significantly to regional and local biodiversity due to their high habitat complexity and a large number of source types. Single sites can be rich or poor in species numbers (α diversity), whilst at the landscape level, the diversity of species (γ diversity) is typically high [6]. On a regional scale, springs host a large number of taxa, and frequently, their study allows for the detection of species new to science or belonging to "Red Lists" [5]. A high species richness on a territorial scale is related to the variety of geological conditions that lead to a wide variation in hydrochemistry, especially in conductivity and pH.

A high geological diversity translates into a high geomorphological and hydrochemical variety in the springs, which in turn is reflected in a high γ diversity [6].

The spring environments are very diverse and often extreme regarding their ecological factors, such as pH, electrolyte content, and temperature. Accordingly, the organisms that populate them and belong to different taxa, evolved various behavioral, biochemical, morphological, and physiological adaptations.

Diatoms are a very diverse and numerous group [7] of eukaryotic unicellular microalgae characterized by a silica cell wall. They are common in inland, transition, and marine waters and also in all terrestrial habitats where some moisture is at least sporadically available. They play a major role among the primary producers in the biosphere (at least 20% of global primary production [8]), and many of them have been documented to have very specific environmental preferences [9]. Their crucial role in the global carbon cycle makes them one of the major players, as well as sentinels of environmental disturbance, in the context of the global change scenario [10,11].

Among the main environmental determinants that control diatom species composition in springs, electrolyte content, discharge, light conditions, current velocity, and algal nutrient concentrations should be mentioned.

Current velocity is very important, especially in rheocrenes. Species found in rivers and springs characterized by a considerable flow may also be present in small rheocrenes if there are microhabitats with relatively strong currents (for example, *Achnanthidium pyrenaicum* (Hust.) H.Kobayasi and *Cocconeis placentula* Ehrenb). On the contrary, some diatom species have adapted to living in environments with an extremely reduced current flow or even in sub-aerial conditions (for example, *Humidophila perpusilla* (Grunow) R.L.Lowe, Kociolek, J.R.Johansen, Van de Vijver, Lange-Bert. et Kopalová, *H. contenta* (Grunow) Lowe, Kociolek, Johansen, Van de Vijver, Lange-Bert. et Kopalová).

In spring ecosystems, diatoms can be used as indicators of a variety of factors with different applications, e.g., nature conservation. The diatom Red List [12] is an important tool to demonstrate their threat status. This allows, on the one hand, a characterization of the ecological integrity and diversity in different types of inland water ecosystems, including an assessment of the threat status of the habitat, and, on the other hand, offers extensive possibilities to monitor the effects of stressors and environmental changes [13]. The proportion of diatom species belonging to threatened categories is a good indicator of the integrity of the source habitat that could decrease due to an increase in nitrates in the groundwater or an alteration of the morphology of the source [13].

Diatoms can also be excellent indicators of flow variability in springs [13]. As already recognized in an index (M) many years ago by Van Dam et al. [14], they live not only in the wetted environment but also in microhabitats that are only periodically (discharge fluctuations) or intermittently (spray zones) wetted. Thus, the composition of their assemblages can provide information on the hydroperiod and flow variability and persistence (e.g., [15]), which is very useful in particular in explorative hydrogeological studies.

Work on diatoms as indicators of flow variability in spring habitats is still rare. Cantonati et al. [13] investigating springs in Berchtesgaden National Park revealed a statistically significant correlation between the Meinzer discharge variability index and the cumulative relative abundance of aerial diatom species (more precisely the sum of the number of species belonging to Van Dam et al.'s [14] M moisture categories 4 and 5 + the number of species listed as aerial "ae" in Hofmann et al. [12]). Even though the relationship was based on a limited number of data, the trend was clear.

More evidence on the overwhelming influence of hydrology-related parameters on diatom communities has been gained for Mediterranean streams (see, e.g., [16] and references therein). One of the hydrology-related parameters was studied by Falasco et al. [17] who concluded that diatoms' resistance mechanisms did not play a significant role in recovery patterns after droughts. Artigas et al. [18] showed that colonization sequences of algae and bacteria in biofilm formation were faster in Mediterranean streams compared to central European ones and that Mediterranean stream biofilms had a larger amount of early-colonizing diatom species (*Ulnaria ulna* (Nitzsch) Compère, *Karayevia clevei* (Grunow) Bukhtiyarova). Furthermore, in their investigation, high rates of polysaccharide production in the extracellular matrix were noted. Calapez et al. [19] and Piano et al. [20] concluded that the replacement of species sensitive to organic pollution with tolerant ones might be the main process in diatom benthic assemblages during droughts.

Calapez et al. [19] also observed no significant difference in evenness or abundance for post-drought diatom assemblages (as compared to pre-drought) whilst diatom quality index values decreased. Falasco et al. [17] concluded that flow intermittency favors pseudaerial and planktic species. At the same time, it threatens endangered diatom Red List species in Mediterranean streams.

Given the key ecological functions that springs have, they play an important role in terms of the effects of climate change on the water balance and in terms of biodiversity. Berchtesgaden National Park recognized the importance of having to perform research to document the effects of climate change on its springs many years ago. Data have now been collected for about twenty-five years and are of fundamental importance for assessing the effects of climate change on the ecosystems. The Ministry of the Environment in Bavaria has financed and supported a project (2018–2020) that regards springs as sentinels of climate change in the two Bavarian national parks. Currently, Steigerwald Nature Park and the UNESCO Rhön Biosphere Reserve are part of this monitoring. Diatoms have been seen to show considerable potential as sensitive indicators of environmental changes both in the Alps and in subarctic regions. They have been used regularly for this purpose in the southeastern Alps (Adamello-Brenta Nature Park) [21] and more sporadically in the northeastern region (Berchtesgaden National Park) [13].

In the frame of the mentioned larger project aimed at using selected springs as sentinel environments of climate change effects, in the present study, we characterize for the first time the diatom communities of the springs of Steigerwald Nature Park (in the following "Steigerwald") and the UNESCO Rhön Biosphere Reserve (in the following "Rhön") and mainly investigate how flow variations, including the extreme situation of desiccation, influence the characteristics and distribution of diatom assemblages in these springs.

2. Materials and Methods

2.1. Study Areas

The Biosphere Reserve Rhön is a UNESCO site, which means that it is a highly protected area. There are more than 700 UNESCO biosphere reserves in the world, 16 of them in Germany, and the Rhön has been part of this framework since 1991. The area covers about 2400 km^2 and has a human population density of 87 inhabitants/km^2; therefore, it is considered a rural or sparsely populated area (Figure 1). The average annual rainfall is between 1100 mm in the mountain areas, and, due to its location in the rain shadow of the mountains, as low as 500 mm in the lower areas. The average annual temperature ranges between 5 to 9 °C, mainly depending on the altitude [22]. A total number of 40 habitat types characterize the area; the most important units are: conical mountains characterized by woods surrounding a central basaltic plateau, steep slopes with limestone grasslands, open meadows without settlements, and vast areas of hedgerows, beech woods, and deciduous forests. Parts of the area are rich in water that emerges on the surface as a spring and collects in small streams where many moisture-loving species find favorable conditions for their development. The Rhön is tripartite from an administrative standpoint, parts of it being included in three German *Länder*: Hesse, Thuringia, and Bavaria. The anterior Rhön north of Bavaria consists of colorful sandstone or *Buntsandstein* (251 to 243 million years old). The reliefs of the anterior Rhön are located between 200 and 600 m a.s.l. Here, the starting rocks in the soil formation are mainly claystone and *Buntsandstein* sandstone. The upper Rhön region has reliefs that reach 600 and 900 m a.s.l. and already has a mid-mountain character. This area is characterized by marine limestone rocks (248–235 million years old) and volcanic rocks more resistant to weathering and erosion. Basalts but also limestones and marl are the most common rocks from which soils originate in the upper Rhön [23].

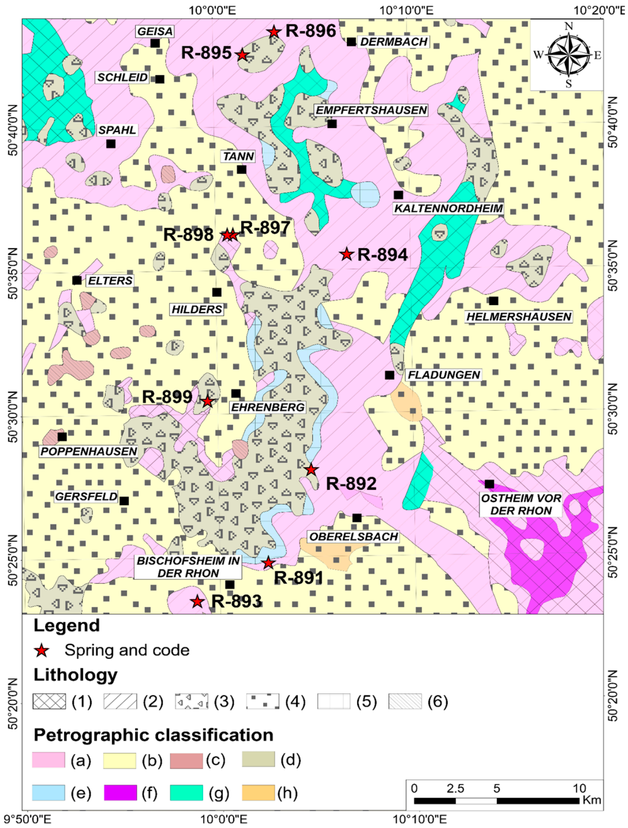


Figure 1. Geological map showing the UNESCO Rhön Biosphere Reserve (Bavaria, Germany) with the location of the springs studied. (1) Mix carbonate rocks, (2) pure carbonate rocks, (3) mafic rocks, (4) sandstone, (5) claystone, and (6) intermediate rocks. (a) Limestone, marlstone, dolomite, sandstone (middle Triassic), (b) sandstone, siltstone, mudstone, conglomerate (lower Triassic), (c) phonolite (Paleogene–Neogene), (d) alkaline basalt, basalt tuff, basanite, tephrite (Paleogene–Neogene), (e) clay, silt, sand, gravel (Miocene), (f) marlstone, gypsum, anhydrite (middle Triassic), (g) mudstone, sandstone, dolomite, marlstone (upper Triassic), (h) sand, gravel (Pleistocene–Holocene). Geological and lithological data source: Federal Institute for Geosciences and Natural Resources (BGR).

The Steigerwald is a middle-elevation mountain range in northern Bavaria (Germany) that is covered by the Steigerwald Nature Park, including several protected areas. The study area is dominated by mixed-broadleaved forests and reaches a height up to 498 m a.s.l. The average annual rainfall is between 700 and 800 mm, and the average annual temperature is around 8.1 °C. The Steigerwald is located in the hydrogeological area of Keuper and Albvorland, which is characterized by clay and sandstone deposits. The selected monitoring sites are all located in the northern half of the Steigerwald (Figure 2) [24].

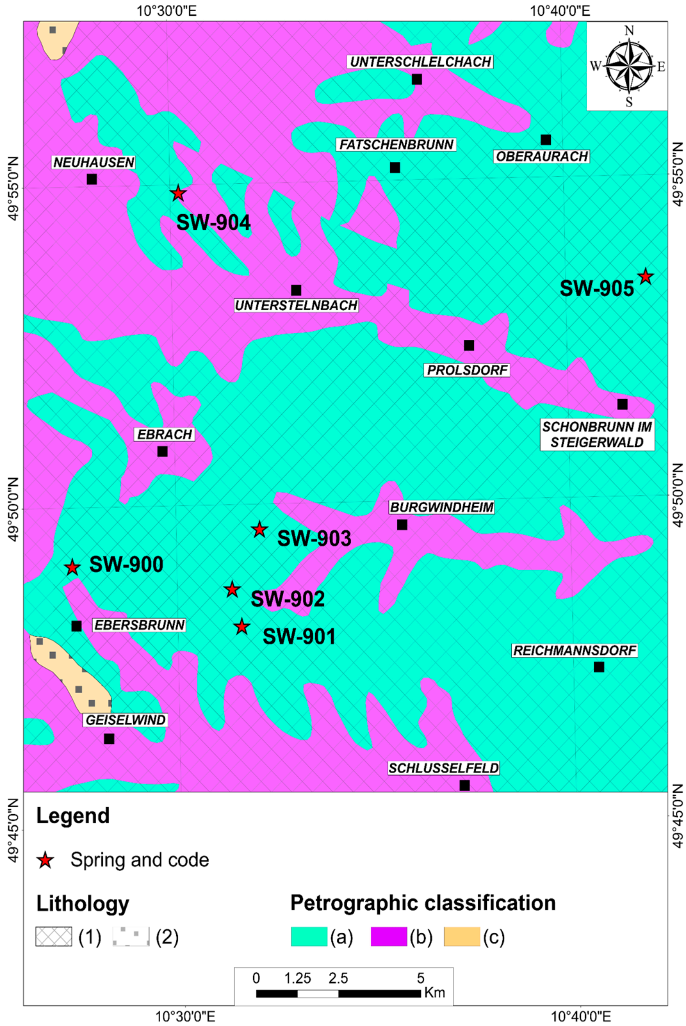


Figure 2. Geological map showing the northern part of the Steigerwald (Bavaria, Germany) with the location of the springs studied. (1) Mix carbonate rocks and (2) sandstone. (a) Mudstone, sandstone, dolomite, marlstone (upper Triassic), (b) marlstone, gypsum, anhydrite (middle Triassic), (c) sand, gravel (Pleistocene–Holocene). Geological data source: Federal Institute for Geosciences and Natural Resources (BGR).

2.2. Field Work and Sampling

The sampling surveys for this study involved 15 springs in total, of which 9 belonged to the Rhön and 6 belonged to the Steigerwald. In the following, the codes used for the springs will report in front of the number that characterizes them: the abbreviation "R" to indicate belonging to the Rhön and "SW" to indicate belonging to the Steigerwald; the same abbreviations may also be used to distinguish the two study areas.

The 15 sampled sources are (codes and identification names from the two projects):

- Steinberg 39 (R891), Gangolfsberg 2 (R892), Kalktuff 1 (R893), Lahrbach 1 (R894), Mannsberg 9 (R895), Hoher Stern 28 (R896), Auersberg 133 (R897), Auersberg 119 (R898), and Buchenstrauch 14 (R899) for the Rhön.
- For the Steigerwald, 80 (SW900), 393 (SW901), 385 (SW902), 353 (SW903), 225 (SW904), and 528 (SW905). The fieldwork/sampling campaign was carried out from 27 June to 2 July 2021. The following parameters were measured: discharge, water temperature, conductivity, pH, and hydrochemistry (main ions and algal nutrients).

2.3. Geology and Hydrogeology

Geological and lithological data used for this work were derived from the Geological Map of Germany 1:1,000,000 and from the 1:5 million International Geological Map of Europe developed by the Federal Institute for Geosciences and Natural Resources (BGR) under the supervision of the Commission of the Geological Map of the World.

The lithological variable was defined to express the main subunits present in the area studied and their influence on the hydrochemistry of the springs studied (details in Table 1).

The springs were classified based on their variation in discharge and quantified using the Meinzer [25] variability index (Rv). This index is a function of the maximum (QM), minimum (Qm), and mean (Qmed) discharge in a hydrological year: $Rv = [(QM - Qm)/Qmed) \times 100]$. Values of $Rv < 25\%$ indicate springs with constant discharge, whereas Rv between 25% and 100% classifies a spring as sub-variable. Higher values ($Rv > 100\%$) identify springs with variable discharge.

2.4. Hydrochemistry

Water samples were collected using polyethylene (PE) bottles cleaned with ultra-pure HNO_3 (Ultrapure grade, Romil, Cambridge, UK) and rinsed with ultra-pure water (Purelab Ultra Analytic, Elga Lab Water, High Wycombe, UK). Samples for major ions and nutrients were kept chilled (ca. 4 °C) in fridge bags until analysis. Hydrochemical analysis followed standard methodology [26]. Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, NO_3^{-}, and SO_4^{2-} were analyzed via ion chromatography (ICS 1500 Dionex Corp., Sunnyvale, CA, USA) and nutrients (N-NO_2^{-}, P-PO_4^{3-}, TP, TN, SiO_4) via standard absorption spectrometry (details in [15]).

2.5. Diatom Sampling, Identification, and Quantification

Diatom assemblages were sampled and treated following Cantonati et al. [15] with specific designation of the spring-head area (=eucrenal), choice of substrata, and sample preparation. Epilithic diatoms were collected by brushing ten stones. The epibryon (=diatoms living on mosses) were collected from the most frequent and abundant bryophytes in each sample location. Epipelic diatoms (=on surface sediment, upper few mm) were sampled using a large bore syringe. The collected materials were digested using hydrogen peroxide, whilst bryophytes were cut into small pieces and digested using the strong acids method [15]. The cleaned material was mounted in Naphrax (refractive index of 1.74). For each sample, a permanent slide was prepared, and 400 valves were counted. All slides were then scanned for taxa with low relative abundances for several hours. All samples (original samples, suspensions of digested material, and permanent mounts) were cataloged and deposited in the collections of the MUSE—Museo delle Scienze (Trento). Observations and counts were conducted with a Zeiss Axioskop 2 at ×1000 magnification (Zeiss, Oberkochen, Germany) equipped with a digital camera Axiocam (Carl Zeiss JSC, Milan, Italy). The most updated taxonomy and nomenclature were applied, and identification reference works were as in Cantonati et al. [15], supplemented by: Cantonati et al. [27], AlgaeBase [28], DiatomBase [29], Diatoms of North America [30], and the Freshwater Diatom Flora of Britain and Ireland [31].

Table 1. Morphological, physical, and chemical characteristics of the 15 springs studied in Steigerwald Nature Park and UNESCO Rhön Biosphere Reserve. In cases where several data were available, mean (in bold) and maximum/minimum values are provided. Shading: 1: Springs exposed to full light but with the presence of tall grass, exposition S, SW, or W. 2: Tree canopy cover, shrubs, rock walls, or other objects, max 25%. 3: Cover from trees, shrubs, rock walls, or other objects, max 50%. 4: Shaded by trees and shrubs, max 75%, but SE, S, SW, or W exposition. 5: Heavily shaded by trees and shrubs, >75%. Claystone (CY): (C)—clay, (S)—sand, (G)—gravel. Pure carbonate rocks (PCR): (L)—limestone, (M)—marlstone, (D)—dolomite. Mix carbonate rocks (MCR): (SS)—sandstone, (MS)—mudstone, (D)—dolomite. Mafic rock (MR): (AB)—alkaline basalt, (BE)—basanite, (T)—tephrite. Sandstone (SS): (SS)—sandstone, (SI)—siltstone, (M)—mudstone. Disch. = Discharge. MVID = Meinzer Variability Index for Discharge.

Spring Code	R-891	R-892	R-893	R-894	R-895	R-896	R-897	R-898	R-899	SW-900	SW-901	SW-902	SW-903	SW-904	SW-905
Coordinates (WGS84)	50.41383 N 10.04189 E	50.46764 N 10.07887 E	50.39182 N 9.98014 E	50.59260 N 10.11219 E	50.7092 N 10.02535 E	50.72227 N 10.05276 E	50.6046915 N 10.0149148 E	50.6046092 N 10.0101308 E	50.5081572 N 9.9913335 E	49.818113N 10.45577 E	49.80184 N 10.52687 E	49.81156 N 10.5231 E	49.82695 N 10.53515 E	49.91474 N 10.50351 E	49.89039 N 10.70048 E
Altitude, (m.a.s.l.)	505	667	603	573	604	628	570	580	722	436	361	348	387	397	319
Shading, (sc. 1–5)	4–5	4–5	4–5	4	4–5	4–5	4–5	4–5	4–5	4	4–5	4–5	4–5	4–5	3
Spring type	Helo	Helo	Rheo	Helo	Rheo	Helo	Helo	Rheo-helo	Helo	Rheo-helo	Rheo	Rheo	Rheo	Rheo	Rheo
Lithology	PCR (L,M,D)	MR (AB,BE,T)	PCR (L,M,D)	PCR (L,M,D)	MR (AB,BE,T)	PCR (L,M,D)	SS (SS,SI,M)	SS (SS,SI,M)	MR (AB,BE,T)	MCR (SS,MS,D)	MCR (SS,MS,D)	MCR (SS,MS,D)	MCR (SS,MS,D)	MCR (SS,MS,D)	MCR (SS,MS,D)
Disch., L s^{-1}	0.6 (1.3/0.4)	1.7 (5.2/0.04)	0.3 (0.6/0.04)	0.4 (0.4/0.3)	0.2 (0.2/0.1)	0.01 (0.02/0.005)	0.1 (0.1/0.01)	0.1 (0.1/0.01)	0.9 (1.6/0.3)	0.15 (0.02/0.3)	0.5 (0.8/0.2)	0.3 (0.6/0.14)	0.2 (0.7/0.006)	0.05 (0.1/0.02)	0.9 (1.5/0.6)
MVID	150	303.5	186.7	25	50	150	90	90	144.4	186.7	120	153.3	347	160	100
Water T, °C	8 (8.2/7.4)	7.3 (7.5/7.1)	7.8 (8.6/7.1)	7.6 (7.6/7.6)	7.6 (7.6/7.6)	N.R.	8.4 (9.8/6.8)	8.6 (10.1/7.3)	8.1 (8.8/7.5)	8.4 (10.7/6.9)	9.2 (9.4/9)	9.05 (9.5/8.7)	9 (11.4/7.3)	8.7 (9.4/8.3)	9.2 (9.3/9.1)
Cond., µS cm^{-1}	345 (433/287)	189 (214/157)	615 (673/559)	315 (351/278)	209 (215/200)	379 (414/343)	447 (471/427)	573 (584/563)	264 (293/236)	88 (91/86)	776 (856/714)	744 (1022/90)	91.5 (100/78)	205 (353/85)	745 (813/690)
pH	7.7 (8/7.5)	7.5 (7.7/7.4)	7.7 (8.1/7.2)	7.7 (7.9/7.4)	8 (8.5/7.5)	7.1 (7.2/7)	8 (8.3/7.5)	7.5 (7.9/7.3)	7.8 (8/7.4)	6.3 (7.1/5.8)	7.7 (7.8/7.5)	7.5 (8/6.1)	6.7 (7.5/6.2)	7.4 (8.1/6.8)	7.7 (7.9/7.4)
Mg^{2+}, mg L^{-1}	8.9	7.8	4.1	6.9	10.4	5.7	6.5	7	3.9	2.9	38.6	15.6	2.3	3.8	46.1
Ca^{2+}, mg L^{-1}	47.1	18	96.5	33.6	17.3	43.4	81	110	39.8	4	94.7	23	3.8	6.4	92
Na^{+}, mg L^{-1}	2.1	2.5	24.6	2.1	5.7	2.2	2.3	2.2	2.2	4	6.1	4.9	2.4	3.3	4.9
K^{+}, mg L^{-1}	0.6	0.6	0.7	<0.5	1.1	<0.5	0.7	1.9	<0.5	0.8	1.6	2.1	1.3	1.3	1.3
Cl^{-}, mg L^{-1}	2.3	2.3	48.5	2.6	3.9	2.9	2.7	3.2	2.2	4	9.4	3.1	2.1	2.9	5.6
NO_3^{-}, mg L^{-1}	1.7	1.7	2.6	5.5	11.5	16.2	5	4.2	2.9	9.4	15.9	4	2.3		
SO_4^{2-}, mg L^{-1}	8.2	8.2	6.2	6.1	14.5	10.7	8.3	8.1	4.9	11	140	41.1	4.5		
PO_4^{3-}, mg L^{-1}	0.069	0.2	<0.03	0.093	0.14	0.04	0.069	0.21	0.14	0.088	0.038	0.092	0.069		
TP, mg L^{-1}	0.028	0.071	<0.010	0.036	0.047	0.023	0.039	0.071	0.047	-	-	-	-		

2.6. Diatom Indicator and Metrics Data Validation with Invertebrates

To validate the diatom indicators and metrics of discharge variability and desiccation identified, invertebrate data available for the springs studied (Rhön and Steigerwald reserves, unpublished) were carefully evaluated. As parameters, we selected invertebrate species richness and the percentage of terrestrial mites. Moreover, considering species and features of the whole invertebrate community, we assigned springs to a dummy variable 0/1, with 0 = stable, 1 = fluctuating discharge (and high desiccation probability).

2.7. Data Processing and Statistical Analyses

A threat status (used also as a measure of rarity) was assigned to all diatom species according to current [12] and previous Red List data [32]. Hofmann et al. [12] provided further ecological attributes (aerial species) used in this study. Preferences of the individual taxa with respect to moisture were obtained from Van Dam et al. [14].

Shannon–Wiener diversity [33] was calculated using a base-2 logarithm. Because a slightly different number of samples were available in Rhön and Steigerwald, we used accumulation curves to estimate the number of species that would be found if sampling effort was the same [34]. The classic method with random permutations of the samples was used to calculate the mean and the standard deviation. Ordination of samples was performed using nonmetric multidimensional scaling (NMDS) and the Bray–Curtis dissimilarity index. In addition, we tested possible differences among factors (e.g., sampling area, Meinzer R) using permutational multivariate analysis of variance based on distance matrices (a.k.a. *Adonis* in the *vegan* package). Multivariate analyses were carried out in the R statistical environment [35] *vegan* package [36].

3. Results

3.1. Samples Processed

The 50 samples processed came from different substrata (stones, bryophytes + algal mucilages, and surface sediment). For each spring type, we collected samples from the three main microhabitats. For some sites only, in particular, Steinberg 39 (R891), Lahrbach 1 (R894), Auersberg 119 (R898), 80 (SW900), and 225 (SW904), samples were taken from an additional substratum consisting of algal mucilages.

The total number of samples used in the valve counting was 19 for Rhön and 12 for Steigerwald. Not all the substrata had a significant number of valves to allow for counting.

Samples were collected from six helocrenes, seven rheocrenes, and two rheo-helocrenes. In some substrata, mainly detritus (surface sediment), diatoms were present with a very low number of individuals that did not allow for a slide count (R891De, R892De, R893De, R894De, R895De, R896De, R897De, R898De, R899De, SW900De, SW901De, SW902De, SW903De, SW904De, SW905De, R893El, R894El, SW904El, SW905El). In four cases, the taxa could at least be photographed and identified at the species level (R895De, R896De, SW904De, R893El).

3.2. The Diatoms Found in the Springs of the Two Nature Preserves

The most frequent (>5 occurrences) and most abundant (>10%) species found in the Steigerwald Nature Park were: *Achnanthidium lineare* W.Sm., *Amphora micra* Levkov, *Eunotia minor* (Kütz.) Grunow, *Meridion circulare* (Grév.) C.Agardh, *Planothidium frequentissimum* (Lange-Bert.) Lange-Bert., *Planothidium lanceolatum* (Bréb. ex Kütz.) Lange-Bert., *Sellaphora nigri* (De Not.) C.E.Wetzel et L.Ector in C.E.Wetzel et al.

The most frequent taxon was *Planothidium lanceolatum* found in 12 out of 13 samples (in one of the samples, its presence had been detected but not counted because the density of the valves was not significant). It is also the most abundant with a maximum relative abundance of 76.7%.

The diatom species found only in 1 substrate out of 13 samples, therefore being less common (frequency = 1 occurrence) and less abundant (with maximum relative abundance = 0.25%), or rare, were: *Amphora lange-bertalotii* var. *tenuis* Levkov et Metzeltin, *Encyonopsis fonticola* (Hust.) Krammer, *Gogorevia exilis* (Grunow) Czarn., *Gomphonema pumilum* var. *pumilum* (Grunow) E.Reichardt et Lange-Bert., *Meridion infirmatum* E.Reichardt, *Nitzschia* cf. *communis* Rabenh., *Pinnularia microstauron* (Ehrenb.) Cleve, *Caloneis lauta* J.R.Carter, *Pinnularia rupestris* Hantzsch, *Sellaphora pupula* (Kütz.) Mereschk., *Stauroneis smithii* Grunow (Figure 3).

In the UNESCO Rhön Biosphere Reserve, the most frequent (>5 occurrences) and most abundant (>10%) species are: *Achnanthidium dolomiticum* Cantonati et Lange-Bert., *Achnanthidium lineare*, *Achnanthidium minutissimum* (Kütz.) Czarn., *Amphora pediculus* (Kütz.) Grunow in A.Schmidt et al., *Caloneis fontinalis* (Grunow) Lange-Bert. et E.Reichardt, *Caloneis lancettula* (Schulz-Danzing) Lange-Bert. et Witkowski, *Gomphonema angustum* C.Agardh, *Humidophila contenta* (Grunow) R.LLowe, Kociolek, Johansen, Van de Vijver, Lange-Bert. et Kopalová, *Humidophila perpusilla* (Grunow) R.L.Lowe, Kociolek, J.R.Johans., Van de Vijver, Lange-Bert. et Kopalová, *Planothidium lanceolatum*, *Psammothidium grischunum* (Wuthrich) Bukht. et Round, *Sellaphora nigri* (Figure 3).

As for the Steigerwald, the most abundant taxon is *Planothidium lanceolatum* with its presence in 22 samples out of 22 sampled (in two substrates, it has been identified but not counted due to the low valve density), while the species with the greatest maximum relative abundance (53%) is *Humidophila perpusilla*, found in 13 samples out of 22.

The less common (frequency = 1 occurrence) and less abundant (maximum relative abundance = 0.3%) species are the following: *Brachysira calcicola* var. *calcicola* Lange-Bert., *Meridion infirmatum*, *Microfissurata paludosa* Cantonati et Lange-Bert., *Navicula cryptotenelloides* Lange-Bert., *Navicula wygaschii* Lange-Bert., *Navicula antonii* Lange-Bert. in Rumrich et al., *Nitzschia pura* Hust., *Stauroneis silvahassiaca* Lange-Bert. et Werum.

3.3. Representation of the Diatom Species Found in the Red List

A table has been processed for the study areas (Table 2). The Red List (RL) threat status of each species [12] is reported both in the 1996 version [37] and in that of 2018. A comparison is reported between the state of the species in 1996 (RL1996) and in 2018 (RL2018); the ecology, according to the RL 2018, is also listed.

The information about the threat status is available for 96 species out of 107 (90%) for Rhön and for 75 species (96%) out of 78 for Steigerwald [12].

A percentage of 41% of the species present in the Rhön samples and 42% of those present in the Steigerwald belong to the Red List categories 2, 3, G, R, V, and D of the Red List; the remaining percentage consists of species not threatened (52 species in Rhön and 42 species in Steigerwald).

The above-mentioned categories 2, 3, G, R, V, and D are represented in Figure 4.

3.4. Ecological Attributes Associated with Discharge Fluctuation and Desiccation

For the identified and counted species (Table 2), the M (moisture) index of Van Dam et al. [14] has also been reported. Data were available for 46 species in Steigerwald (59%) and 54 species in Rhön (50%).

In Rhön, for 12 species, the moisture index M was =4, so they are frequent in wet areas or where water is temporarily not available: *Adlafia minuscula, Caloneis tenuis, Halamphora normanii, Humidophila brekkaensis, Humidophila contenta, Mayamaea fossalis* var. *fossalis, Navicula tenelloides, Nitzschia hantzschiana, Pinnularia obscura, Sellaphora stroemii, Stauroneis parathermicola, Stauroneis thermicola.*

Both in Rhön and in Steigerwald, 2 species have index M = 5 (they live almost totally outside water): *Adlafia bryophila*, *Humidophila perpusilla*.

Table 2. Diatom taxa list grouped into microhabitat/substrate type: bryophytes (Ep, epiphytic), lithic material (El, epilithic), "*Algenwatten*"/algal slime (Ma, supplementary sample) with diatom Red List ecological groups and Van Dam moisture index information. M.A.—maximum relative abundance; N.O.—occurrence; RL18—Red List of diatoms for Germany 2018 [12]: RL96—previous version of Red List for Germany [32]. Red List categories [12]: 1—threatened with extinction, 2—strongly threatened, 3—threatened, G—threat of unknown extent, R—extremely rare, V—declining, D—data insufficient, *—not threatened, **—surely not threatened, ⧫—not evaluated. ** ~— the taxonomic concept in RL18 overlaps with the one that was used in RL96. Diatoms' threat state evolution comparing RL18 and RL96: =—threat status remained the same, -—threat status improved (the species is less threatened), +—threat status worsened (the species is more threatened). Ecology according to Hofmann et al. [12]: ae—aerial, o—oligotraphentic, oc—oligotraphentic carbonate, od—oligotraphentic distrophic, eu—eutraphentic to tolerant, ?—unknown. M—moisture index according to Van Dam [Van Dam et al. [14]]: 1—almost never occurring outside water bodies, 2—mainly occurring in water bodies, sometimes in wet places, 3—mainly occurring in water bodies, also rather regularly on wet and moist places, 4—mainly occurring on wet and moist or temporarily dry places, 5—nearly exclusively occurring outside water bodies, -—the species was present in the old Van Dam et al. [14] list but that a value of the index is not provided for the species. According to the microhabitat where they were found for less than 50% of sampling sites—"x", 50% and more of sampling sites—"xx", 80% and more of sampling sites—"xxx".

Taxa	M.A.	N.O.	RL18	RL96	Evolution	Ecology	M	Ep	El	Ma
Achnanthidium affine (Grunow) Czarn.	7.3%	8	*	*	=	?	-	x	x	xx
Achnanthidium dolomiticum Cantonati et Lange-Bert.	19.3%	20	2	-		?		xxx	xx	x
Achnanthidium gracillimum (Meister) Lange-Bert. in Krammer et Lange-Bert.	0.50%	1	2	3	-	oc	-	x		
Achnanthidium lineare W.Sm.	51.8%	24	G	⧫°		eu	-	xx	xxx	xx
Achnanthidium minutissimum (Kütz.) Czarn.	24.00%	14	*	**°	=	?	3	xx	x	xx
Achnanthidium pfisteri Lange-Bert.	7.50%	7	D	-		?		xx	xx	
Achnantidium polonicum Van de Vijver, Wojtal, Morales et L.Ector	2.00%	1								x
Achnanthidium subatomus (Hust.) Lange-Bert.	1.00%	1	V	*	–	od	1	x		
Adlafia bryophila (Petersen) Lange-Bert. in Moser et al.	3.00%	6	*	V	+	?	5	x	x	x
Adlafia langebertalotii Monnier et L.Ector	0.25%	2	*	–		?			x	x
Adlafia minuscula (Grunow) Lange-Bert. in Lange-Bert. et Genkal	2.00%	10	*	*	=	?	4	x	x	x
Amphora indistincta Levkov	3.50%	3	*	-		?			x	x
Amphora lange-bertalotii var. *tenuis* Levkov et Metzeltin	0.25%	2	D	-		o			x	
Amphora micra Levkov	49.00%	23						xx	xx	xx
Amphora pediculus (Kütz.) Grunow in A.Schmidt et al.	39.3%	15	*	**	=	?	3	xx	xx	xx
Brachysira calcicola var. *calcicola* Lange-Bert.	0.25%	1	2	⧫°		oc		x		
Caloneis constans E.Reichardt	4.25%	2	R		–	oc		x		

Table 2. *Cont.*

Taxa	M.A.	N.O.	RL18	RL96	Evolution	Ecology	M	Ep	El	Ma
Caloneis fontinalis (Grunow) Lange-Bert. et Reichardt	15.3%	7	*	–		?		x		x
Caloneis lancettula (Schulz-Danzing) Lange-Bert. et Witkowski	43.5%	9	*	♦°		eu		x	x	
Caloneis lauta J.R.Carter	0.25%	1	G	G	=	o	1	x		
Caloneis tenuis (W.Greg.) Krammer	0.50%	2	3	G		o	4	x	x	
Caloneis vasileyevae Lange-Bert., Genkal et Vekhov	2.00%	2	R	-		?		x		
Cymbella compacta Østrup	0.75%	1	*	*	=	eu		x		
Cymbopleura austriaca (Grunow) Krammer	0.50%	1	2	V	–	ae/oc		x		
Cocconeis euglypta Ehrenb.	4.80%	15	*	**	=	?	2	xx	x	x
Cocconeis lineata Ehrenb.	9.50%	11	*	**	=	?	2	x	x	x
Cocconeis pseudolineata (Geitler) Lange-Bert.	7.30%	8	*	D		?	2	x	x	
Cocconeis pseudothumensis E.Reichardt	1.00%	2	G	3		oc	3	x	x	
Craticula minusculoides (Hust.) Lange-Bert.	0.50%	1	*	*	=	eu	2	x		
Craticula vixnegligenda Lange-Bert.	0.50%	1	G	R	-	o		x		
Denticula tenuis Kütz.	1.80%	4	*	*	=	o	3	x	x	
Diploneis fontanella Lange-Bert.	1.30%	2	G	-		o		x		
Diploneis krammeri Lange-Bert. et E.Reichardt	0.50%	2	V	♦°		oc		x		
Diploneis separanda Lange-Bert.	1.00%	3	D	♦°		oc		x	x	
Encyonopsis falaisensis (Grunow) Krammer	1.00%	1	G	G	=	o	3	x		
Encyonopsis fonticola (Hust.) Krammer	0.25%	1						x		
Eunotia boreoalpina Lange-Bert. et Nörpel-Schempp in Lange-Bert. et Metzeltin	0.50%	1	G	-		od	2	x		
Eunotia minor (Kütz.) Grunow	14.2%	5	V	*	–	?	-	x		xxx
Eunotia paratridentula Lange-Bert. et Kulikovskiy	1.50%	1	3	*	–	od		x		
Eunotia soleirolii (Kütz.) Rabenh.	7.25%	3	G	G	=	od	1	x	x	
Frustulia vulgaris (Thwaites) De Toni	0.25%	2	*	**	=	?	3		x	
Gogorevia exilis (Grunow) Czarn.	0.25%	1					2	x		
Gomphonema angustum C.Agardh	18.00%	12	G	V	-	oc	-	xx	x	x
Gomphonema cuneolus E.Reichardt	10.00%	1	G		-	oc			x	
Gomphonema cymbelliclinum E.Reichardt et Lange-Bert.	3.00%	1	*	-		?		x		
Gomphonema elegantissimum E.Reichardt et Lange-Bert.	22.3%	4	*	-		oc		x	x	
Gomphonema exilissimum (Grunow) Lange-Bert. et Reichardt	1.00%	1	V	V	=	od	-			x

Table 2. *Cont.*

Taxa	M.A.	N.O.	RL18	RL96	Evolution	Ecology	M	Ep	El	Ma
Gomphonema extentum E.Reichardt et Lange-Bert.	4.3%	4	*	**	=	hal		x	x	xx
Gomphonema hebridense W.Greg.	0.50%	1	V	V	=	?		x		
Gomphonema innocens E.Reichardt	0.75%	2	*	-		?		x		
Gomphonema micropus Kütz.	4.00%	10	*	♦°		?	3	x	x	x
Gomphonema minutum (C.Agardh) C.Agardh	3.25%	1	*	**	=	eu	-	x		
Gomphonema productum (Grunow) Lange-Bert. et Reichardt in Lange-Bert.	2.00%	6	V	V	=	?		x	x	
Gomphonema pseudotenellum Lange-Bert. in Krammer et Lange-Bert.	1.50%	2	G	3		o		x		x
Gomphonema pumilum (Grunow) E.Reichardt et Lange-Bert.	9,75%	4	*	♦°		?	-		x	
Gomphonema sarcophagus W.Greg.	5.00%	3	V	V	=	?	3		x	x
Gomphonema subclavatum (Grunow) Grunow	3.30%	6	*	♦°		?	3	x	x	x
Gomphonema utae Lange-Bert. et E.Reichardt in Reichardt	16.8%	4	*	D		?		x	x	xx
Gomphonema varioreduncum Jüttner, L.Ector, E.Reichardt, Van de Vijver et E.J.Cox in Jüttner et al.	0.50%	2	D	–		od		x		
Grunowia sinuata (Thwaites) Rabenh.	0.50%	2							x	x
Halamphora normanii (Rabenh.) Levkov	7.00%	5	D	♦°		ae	4	x		
Hantzschia calcifuga E.Reichardt et Lange-Bert.	1.25%	1	D	-		od		x		
Humidophila brekkaensis (Petersen) R.LLowe et al.	1.00%	6	*	V	+	ae		x	x	x
Humidophila contenta (Grunow) R.LLowe, Kociolek, Johansen, Van de Vijver, Lange-Bert. et Kopalová	12.2%	12	D	♦°		ae	4	xx	x	
Humidophila perpusilla (Grunow) R.L.Lowe, Kociolek, J.R.Johans., Van de Vijver, Lange-Bert. et Kopalová	53.00%	22	*	**	=	ae/o	5	xxx	xx	x
Karayevia clevei (Grunow) Bukht.	6.80%	3	*	*	=	eu	1	x	x	
Kobayasiella subtilissima (Cleve) Lange-Bert.	0.75%	1	2	2	=	od	3	x		
Luticola acidoclinata Lange-Bert.	0.50%	1	G	D		od		x		
Luticola goeppertiana (Bleisch) D.G.Mann	0.50%	1	*	♦°		eu	3	x		
Mayamaea fossalis var. *fossalis* (Krasske) Lange-Bert.	2.50%	4	*	*	=	?	4	x	x	x
Mayamaea permitis (Hust.) K.Bruder et Medlin	5.75%	6	*	**	=	eu	3			x
Meridion circulare (Grév.) C.Agardh	27.3%	18	*	**	=	?	1	x	xx	xx
Meridion constrictum Ralfs	1.50%	1	*	**	=	o	2	x	x	
Meridion infirmatum E.Reichardt	0.25%	2							x	

Table 2. *Cont.*

Taxa	M.A.	N.O.	RL18	RL96	Evolution	Ecology	M	Ep	El	Ma
Microfissurata paludosa Cantonati et Lange-Bert.	0.25%	1							x	
Navicula antonii Lange-Bert. in Rumrich et al.	2.25%	1	*	**	=	eu	-			x
Navicula cryptocephala Kütz.	0.50%	3	*	** ~	=	eu	2	x	x	x
Navicula cryptotenelloides Lange-Bert.	0.25%	1	*	**	=	?			x	
Navicula exilis Kütz.	0.75%	3	3	G		od	-	x	x	
Navicula gregaria Donkin	1.75%	1	*	**	=	?	5		x	
Navicula moenofranconica Lange-Bert.	0.75%	1	3	3	=	?		x		
Navicula notha J.H.Wallace	2.25%	3	2	G		od	2	x	x	
Navicula tenelloides Hust.	1.00%	4	*	*	=	eu	4	x	x	
Navicula tripunctata (O.F.Müll.) Bory	1.25%	4	*	**	=	eu	3	x	x	x
Navicula wygaschii Lange-Bert.	0.25%	1	G	-		oc			x	
Nitzschia acidoclinata Lange-Bert.	4.25%	3	V	*	–	?	3	x		x
Nitzschia alpina Hust.	1.00%	3	3	G		o	-	x	x	
Nitzschia cf. *communis* Rabenh.	0.25%	1	*	**	=	eu	5		x	
Nitzschia dissipata (Kütz.) Grunow	1.50%	3	*	**	=	eu	3	x	x	
Nitzschia fonticola Grunow in Cleve et Möller	0.75%	3	*	**	=	eu	1	x	x	
Nitzschia hantzschiana Rabenh.	2.25%	9	G	*	–	?	4	x	x	
Nitzschia intermedia Hantzsch in Cleve et Grunow	2.00%	2	*	*	=	eu	1		x	
Nitzschia linearis (C.Agardh) W.Sm.	2.50%	10	*	**	=	eu	3	x	x	x
Nitzschia perminuta (Grunow) H.Peragallo	1.00%	5	*	*	=	?	3	x	x	x
Nitzschia pura Hust.	0.25%	1	*	*°	=	o	-		x	
Odontidium hyemale (Roth) Kütz.	1.00%	1					2		x	
Odontidium mesodon (Ehrenb.) Ralfs	3.00%	5					2	x	x	x
Orthoseira roeseana (Rabenh.) O'Meara	2.00%	1	D	V		ae		x		
Pinnularia microstauron (Ehrenb.) Cleve	0.25%	1	V	V	=	od	7	x		
Pinnularia obscura Krasske	1.00%	1	*	**	=	ae	4		x	
Pinnularia perirrorata Krammer	1.75%	3	*		–	od		x	x	
Pinnularia rupestris Hantzsch	0.25%	1	G	♦° ~		od	1		x	
Pinnularia schoenfelderi Krammer	1.00%	1	G	G	=	od		x		

Table 2. *Cont.*

Taxa	M.A.	N.O.	RL18	RL96	Evolution	Ecology	M	Ep	El	Ma
Planothidium dubium (Grunow) Round et Bukht.	0.50%	1	*	*	=	eu			x	
Planothidium frequentissimum (Lange-Bert.) Lange-Bert.	16.2%	27	*	**	=	eu	-	xxx	xx	xxx
Planothidium lanceolatum (Bréb. ex Kütz.) Lange-Bert.	76.1%	35	*	**	=	?	3	xxx	xxx	xxx
Planothidium reichardtii Lange-Bert. et Werum in Werum et Lange-Bert.	5.00%	9	D	-		?		x	x	x
Planothidium rostratoholoarcticum Lange-Bert. et Bąk	0.50%	2	*	D		eu	3	x		x
Psammothidium grischunum (Wuthrich) Bukht. et Round	31.00%	14	V	*	-	?	-	xx	x	xx
Psammothidium subatomoides (Hust.) Bukht. et Round	12.7%	8	G	G	=	od	1	x	x	x
Reimeria sinuata (W.Greg.) Kociolek et Stoermer	2.50%	2	*	♦°		?	3		x	
Rhoicosphenia abbreviata (C.Agardh) Lange-Bert.	6.50%	3	*	**	=	eu	2		x	
Rhoicosphenia tenuis Levkov et T.Nakov	11.2%	1							x	
Sellaphora atomoides (Grunow) Wetzel et Van de Vijver	4.75%	8						x	x	
Sellaphora nigri (De Not.) C.E.Wetzel et L.Ector in C.E.Wetzel et al.	27.7%	28					3	xxx	xxx	xxx
Sellaphora pupula (Kütz.) Mereschk.	0.25%	1	D	**°		eu	4		x	
Sellaphora pseudopupula (Krasske) Lange-Bert.	1.50%	1	G	G	=	od		x		
Sellaphora saugerresii (Desm.) C.E.Wetzel et D.G. Mann	5.50%	11					3	xx	x	x
Sellaphora seminulum (Grunow) D.G.Mann	6.00%	4	*	**	=	eu	3	x	x	x
Sellaphora stroemii (Hust.) D.G.Mann	1.30%	2	2	3	-	oc	4	x	x	
Stauroneis kriegeri R.M.Patrick	0.50%	1	*	*	=	?	4		x	
Stauroneis parathermicola Lange-Bert.	0.50%	1	*	–		?			x	
Stauroneis separanda Lange-Bert. et Werum	0.50%	1	V	-		oc			x	
Stauroneis silvahassiaca Lange-Bert. et Werum	0.25%	1	D	–		od		x		
Stauroneis smithii Grunow	0.50%	3	R	♦°		?	3	x	x	
Stauroneis thermicola (J.B.Petersen) Lund	2.50%	5	*	*	=	ae	4	x	x	
Staurosirella pinnata (Ehrenb.) D.M.Williams et Round	26.5%	3	*	**	=	?	7	x	x	
Surirella terricola Lange-Bert. et Alles	0.75%	2	D	*		ae		x	x	

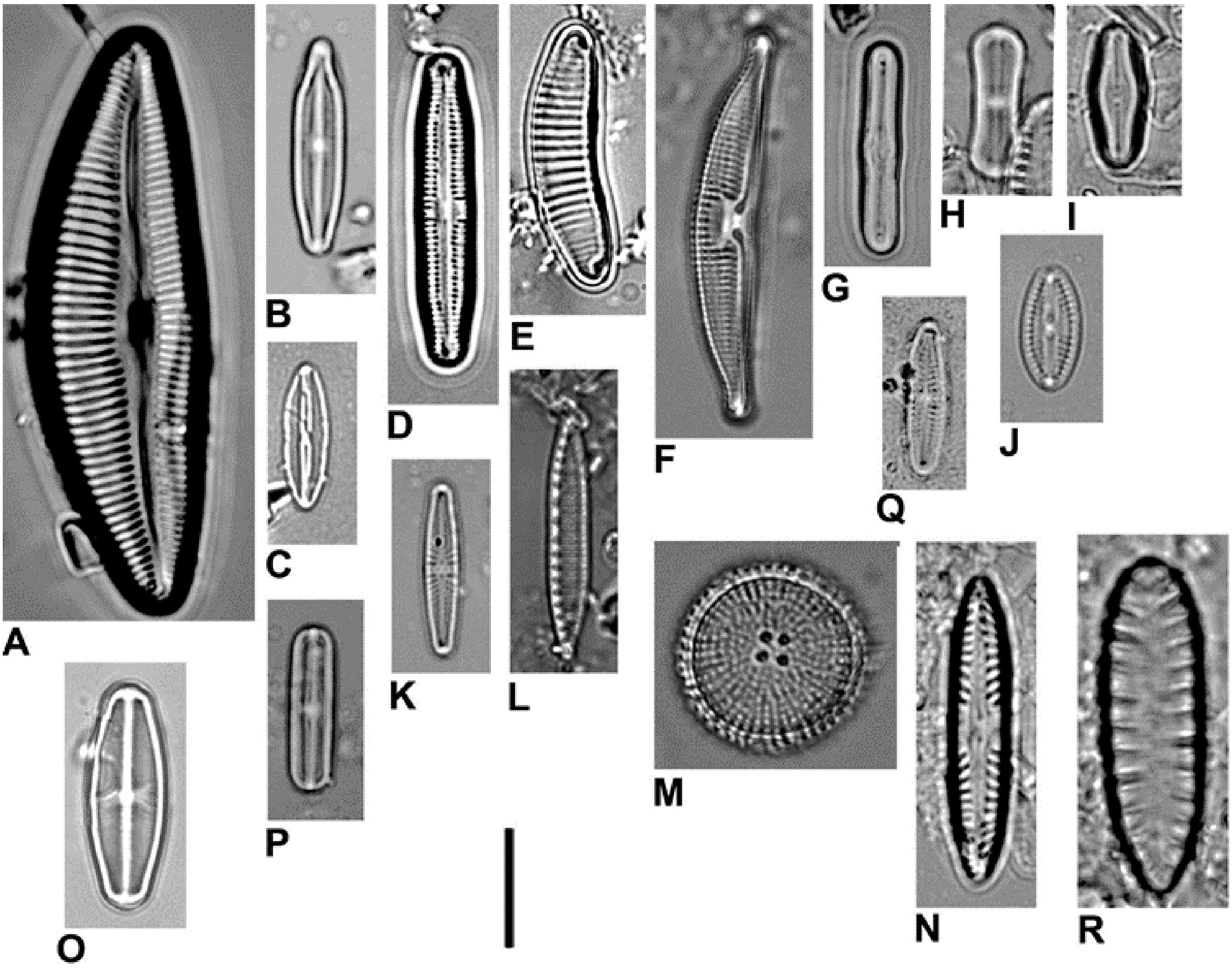


Figure 3. Light micrographs of the most abundant aerial diatom species recorded in the Rhön Biosphere Reserve and Steigerwald Nature Park in the present study according to the moisture index of Van Dam et al. [14]. (**A**) *Cymbopleura austriaca* (SW905Ep), (**B**) *Adlafia bryophila* (R895Ep), (**C**) *Adlafia minuscula* (R898Ma), (**D**) *Caloneis tenuis* (SW901Ep), (**E**) *Eunotia minor* (SW903Ep), (**F**) *Halamphora normanii* (SW905Ep), (**G**) *Humidophila brekkaensis* (R895El), (**H**) *Humidophila contenta* (SW905Ep), (**I**) *Humidophila perpusilla* (SW900El), (**J**) *Mayamaea fossalis* var. *fossalis* (R895Ep), (**K**) *Navicula tenelloides* (R895Ep), (**L**) *Nitzschia hantzschiana* (SW905Ep), (**M**) *Orthoseira roseana* (R895Ep), (**N**) *Pinnularia obscura* (R891Ep), (**O**) *Sellaphora pupula* (SW901Ep), (**P**) *Sellaphora stroemii* (R897El), (**Q**) *Stauroneis parathermicola* (R898El), (**R**) *Surirella terricola* (R893Ep). Scale bar: 10 µm.

In Steigerwald, 10 species have index M = 4: *Adlafia minuscula*, *Caloneis tenuis*, *Eunotia minor*, *Halamphora normanii*, *Humidophila contenta*, *Mayamaea fossalis* var. *fossalis*, *Navicula tenelloides*, *Nitzschia* cf. *communis*, *Nitzschia hantzschiana*, *Stauroneis thermicola*.

Data about the M "moisture" index 4 or 5 as a possible metric of interest are available for 15% of species in SW and 13% in R.

A total number of eight species found in Rhön are identified as aerial "ae" according to Hofmann et al. [12] (*Halamphora normanii*, *Humidophila brekkaensis*, *H. contenta*, *H. perpusilla*, *Orthoseira roeseana*, *Pinnularia obscura*, *Stauroneis thermicola*, *Surirella terricola*).

In Steigerwald, five species are classified as "ae": *Cymbopleura austriaca*, *Halamphora normanii*, *Humidophila contenta*, *Humidophila perpusilla*, *Stauroneis thermicola*.

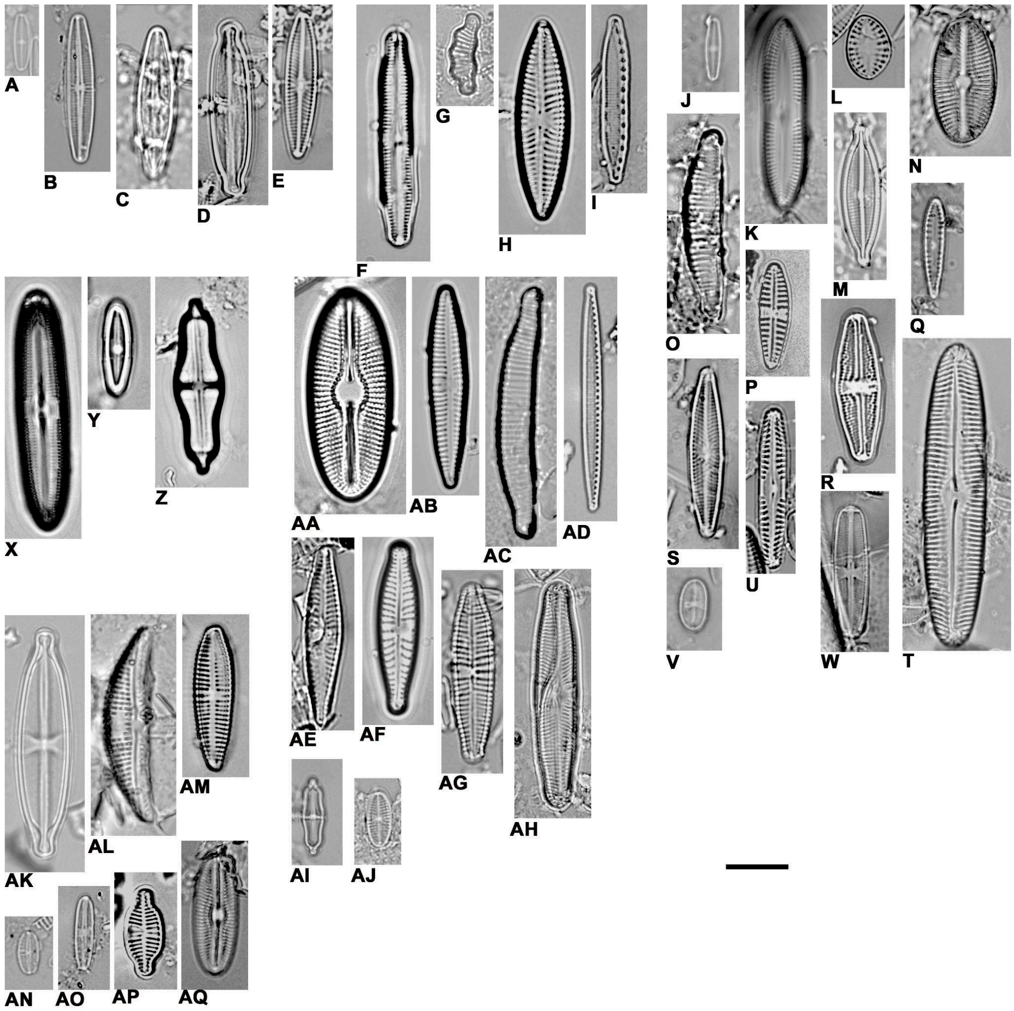


Figure 4. Light micrographs of the diatom Red List recorded in the Rhön Biosphere Reserve and Steigerwald Nature Park. (**A**) *Achnanthidium dolomiticum*-(2)-(R891El), (**B**) *Achnanthidium gracillimum*-(2)-(R896Ep), (**C**) *Brachysira calcicola* var. *calcicola*-(2)-(R897Ep), (**D**) *Kobayasiella subtilissima*-(2)-(R898Ep), (**E**) *Navicula notha*-(2)-(R891Ep), (**F**) *Caloneis tenuis*-(3)-(R892Ep), (**G**) *Eunotia paratridentula*-(3)-(SW903Ep), (**H**) *Navicula exilis*-(3)-(R891Ep), (**I**) *Nitzschia alpina*-(3)-(R895El), (**J**) *Achnanthidium lineare*-(G)-(SW902El), (**K**) *Caloneis lauta*-(G)-(SW904Ep), (**L**) *Cocconeis pseudothumensis*-(G)-(R892Ep), (**M**) *Craticula vixnegligenda*-(G)-(R892Ep), (**N**) *Diploneis fontanella*-(G)-(R891Ep), (**O**) *Eunotia soleirolii*-(G)-(SW903Ep), (**P**) *Gomphonema angustum*-(G)-(SW905Ep), (**Q**) *Gomphonema pseudotenellum*-(G)-(R894Ep), (**R**) *Luticola acidoclinata*-(G)-(R892Ep), (**S**) *Navicula wygaschii*-(G)-(R896El), (**T**) *Pinnularia rupestris*-(G)-(SW902El), (**U**) *Pinnularia schoenfelderi*-(G)-(SW900Ma), (**V**) *Psammothidium subatomoides*-(G)-(SW900El), (**W**) *Sellaphora pseudopupula*-(G)-(SW900Ma), (**X**) *Caloneis constans*-(R)-(R901Ep), (**Y**) *Caloneis vasileyevae*-(R)-(R897Ep), (**Z**) *Stauroneis smithii*-(R)-(R892Ep), (**AA**) *Diploneis krammeri*-(V)-(R892Ep), (**AB**) *Gomphonema hebridense*-(V)-(R892Ep), (**AC**) *Eunotia minor*-(V)-(SW904Ma), (**AD**) *Nitzschia acidoclinata*-(V)-(SW901Ep), (**AE**) *Gomphonema exilissimum*-(V)-(R891Ma), (**AF**) *Gomphonema productum*-(V)-(R892Ep), (**AG**) *Gomphonema sarcophagus*-(V)-(R891Ma), (**AH**) *Pinnularia microstauron*-(V)-(SW904Ep), (**AI**) *Stauroneis separanda* (V)-(R898Ma), (**AJ**) *Psammothidium grischunum*-(V)-(R893Ep), (**AK**) *Stauroneis silvahassiaca*-(D)-(R895Ep), (**AL**) *Amphora lange-bertalotii* var. *tenuis*-(D)-(SW901El), (**AM**) *Gomphonema varioreduncum*-(D)-(R892Ep), (**AN**) *Achnanthidium pfisteri*-(D)-(SW902El), (**AO**) *Sellaphora atomoides*-(D)-(R894Ep), (**AP**) *Planothidium reichardtii*-(D)-(R894Ep), (**AQ**) *Diploneis separanda*-(D)-(R897Ep). Scale bar: 10 µm.

3.5. Teratological Diatom Specimens

Teratological (deformed) specimens of several species were relatively frequent in the studied samples and are presented in Figure 5.

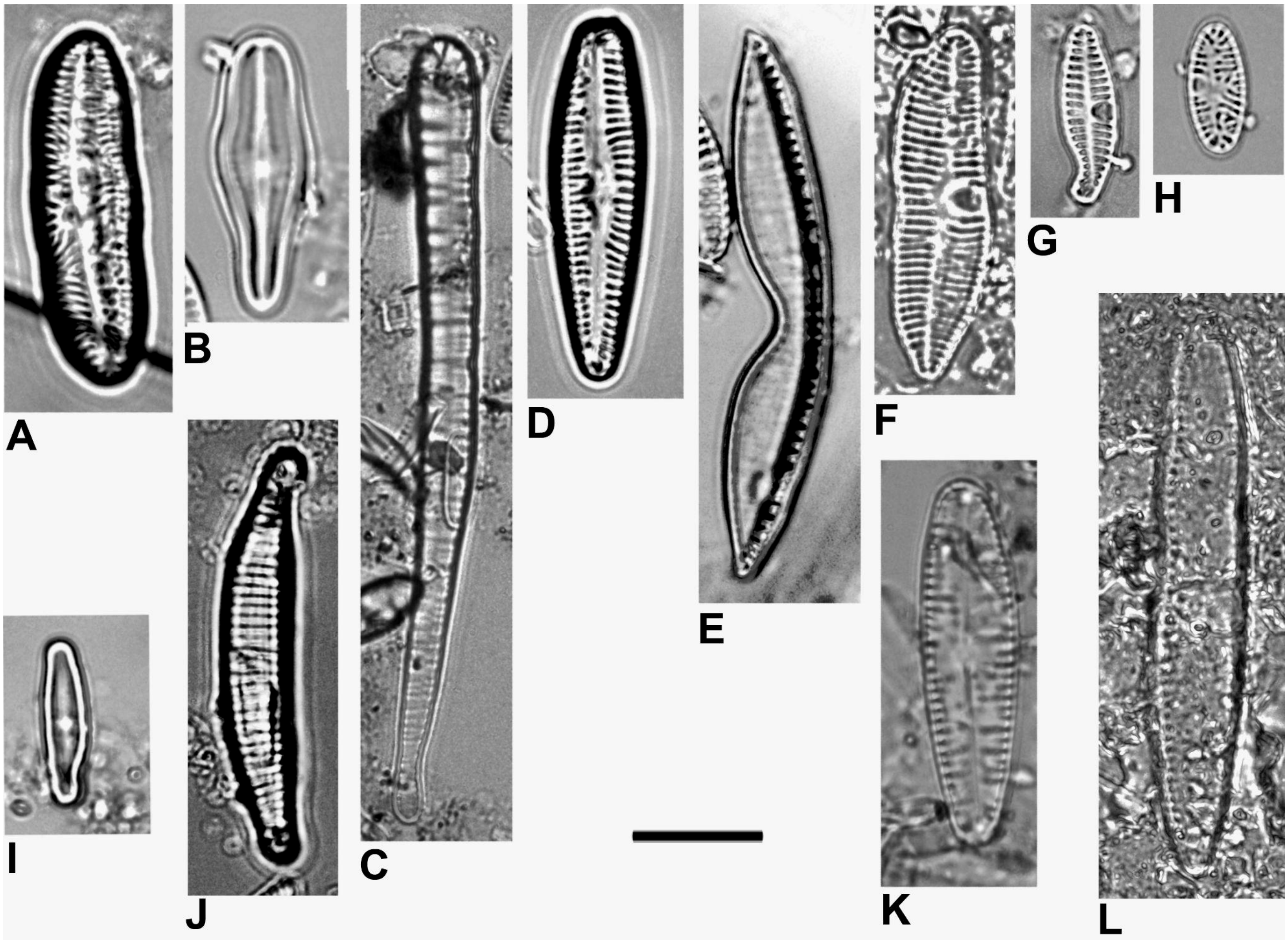


Figure 5. Light micrographs of the most common teratologies found in the present study. (**A**) unidentified teratological diatom form (R892Ep), (**B**) *Humidophila perpusilla* (R892Ep), (**C**) *Meridion circulare* (R891El), (**D**) *Gomphonema* sp. (R892Ep), (**E**) *Nitzschia* cf. *intermedia* (R892 El), (**F**) *Planothidium lanceolatum* (R898Ep), (**G**,**H**) *Planothidium frequentissimum* (SW900Ep, R898Ep), (**I**) *Achnanthidium minutissimum* (R899Ep), (**J**) *Eunotia minor* (SW903Ep), (**K**) *Gomphonema* sp. (R891El), (**L**) *Nitzschia* cf. *communis* (SW901El). Scale bar: 10 µm.

Figure 5 shows a series of teratological forms encountered during the species identification. Morphological anomaly can be due to several factors: heavy metals [38], pesticides, and osmotic stress but also physical stress [39,40]: Some interdependent environmental factors, such as temperature, brightness, and desiccation, may have an influence on silica deposition, resulting in the development of teratological forms. The physical disturbance created by the intermittence of the springs studied can also act on the morphology of these algae. Therefore, the teratology found in some springs is also evidence that strengthens the fact that such sites are affected by intermittent flow. Teratological forms were found in nine out of fifteen sources. Out of these, SW900 and SW905 are subject to seasonal desiccation, and R891, R898, R895, and R896 are characterized by a very low flow rate. In R899, a teratological form of *Achnanthidium minutissimum* of type CLT (cymbelliclinum-like teratology) has been found. This teratology type is the most frequently found in the above-mentioned work [38], and the species is described as rather tolerant to physical stress.

3.6. Results of Preliminary Statistical Analyses

Preliminary statistical analysis (necessary for further data processing) showed that the assemblages found in the two nature preserves did not differ significantly (Figure 6), diatom species richness and Shannon diversity did not differ in a statistically significant way between epilithic (El) and epiphytic (Ep) samples (Figure 7), species richness would be similar if the same number of samples were collected in both protected areas (Figure 8), springs with high tendency to desiccation had lower discharge (Figure 9), and the percentage of terrestrial mites and the Meinzer index for discharge were not significantly correlated (Figure 10).

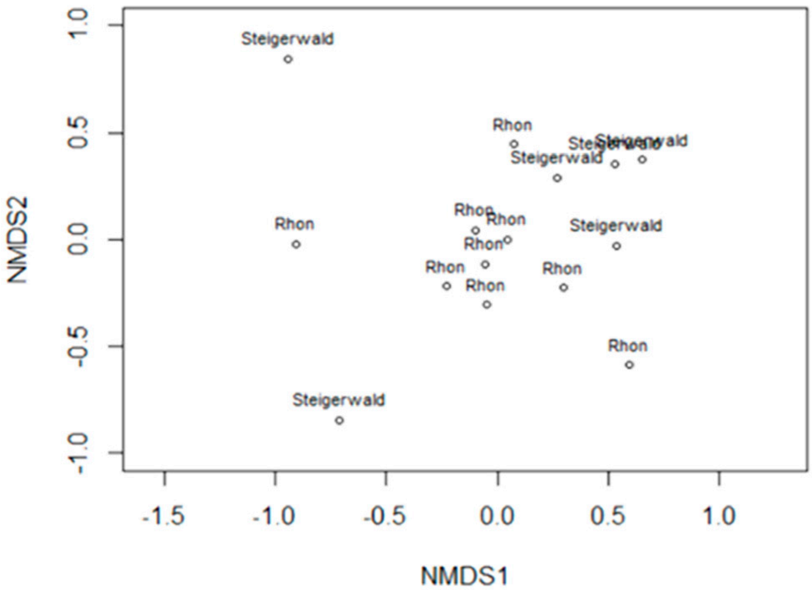


Figure 6. Ordination of the diatom samples collected in Rhön and Steigerwald using the NMDS. The assemblages found in the two nature reserves did not differ significantly after multivariate analysis of variance.

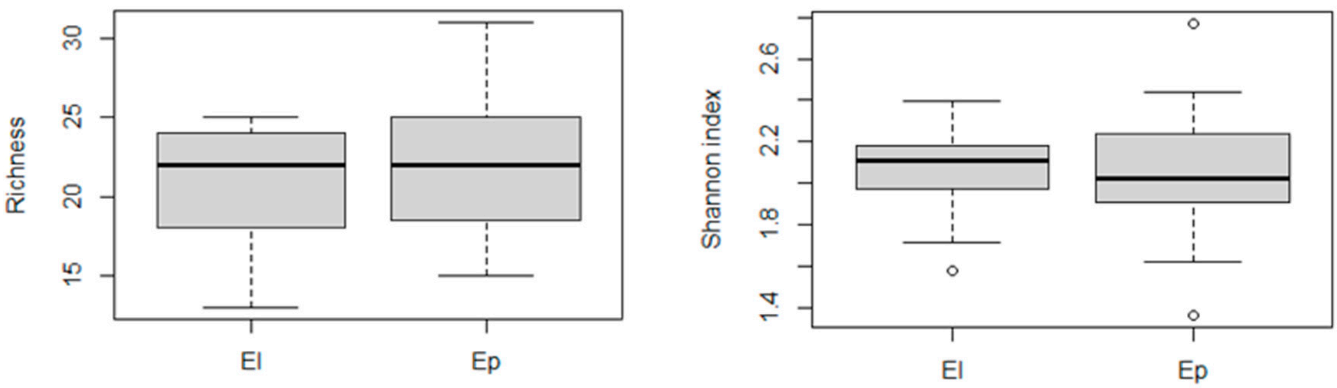


Figure 7. Boxplots of species richness and Shannon diversity of diatoms in epilithic (El) and epiphytic (Ep) samples. Median (bold line), first and third quartile (box), and minimum and maximum (whisker) are shown.

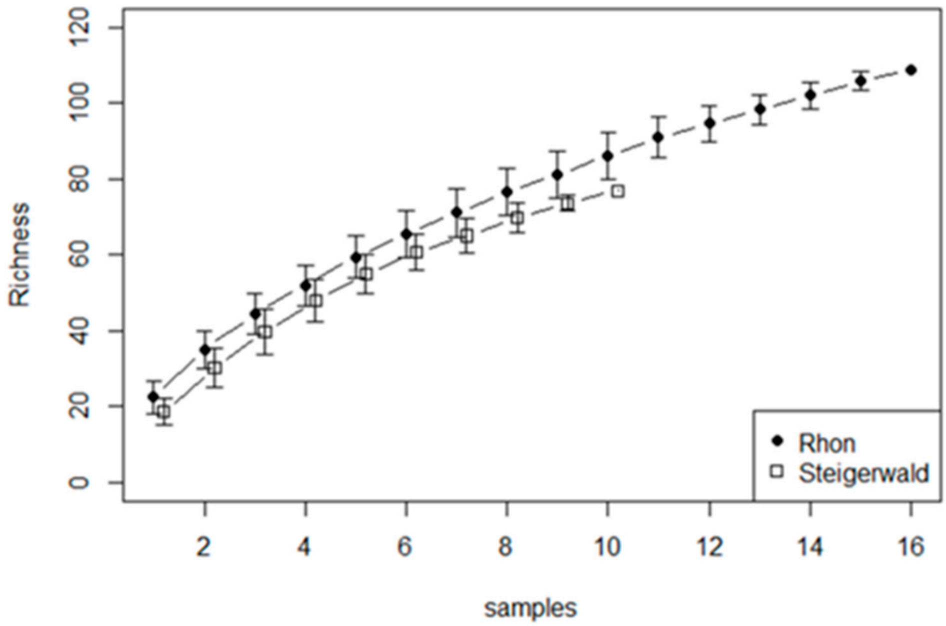


Figure 8. Species accumulation curves for the two protected areas. Species richness would be similar if the same number of samples were collected in both areas.

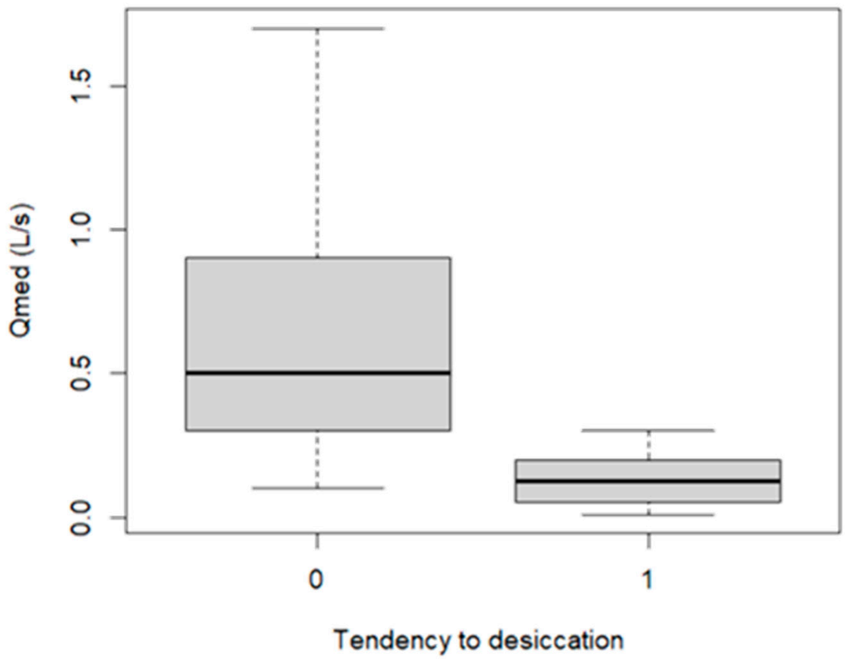


Figure 9. Boxplots of the average discharge (Qmed) found in the springs with (1) or without (0) tendency to desiccation (intermittent vs. perennial). The classification between the two hydrological types was based on the percentage of terrestrial mites found in the springs. See methods for further details. Median (bold line), first and third quartile (box), and minimum and maximum (whisker) are shown.

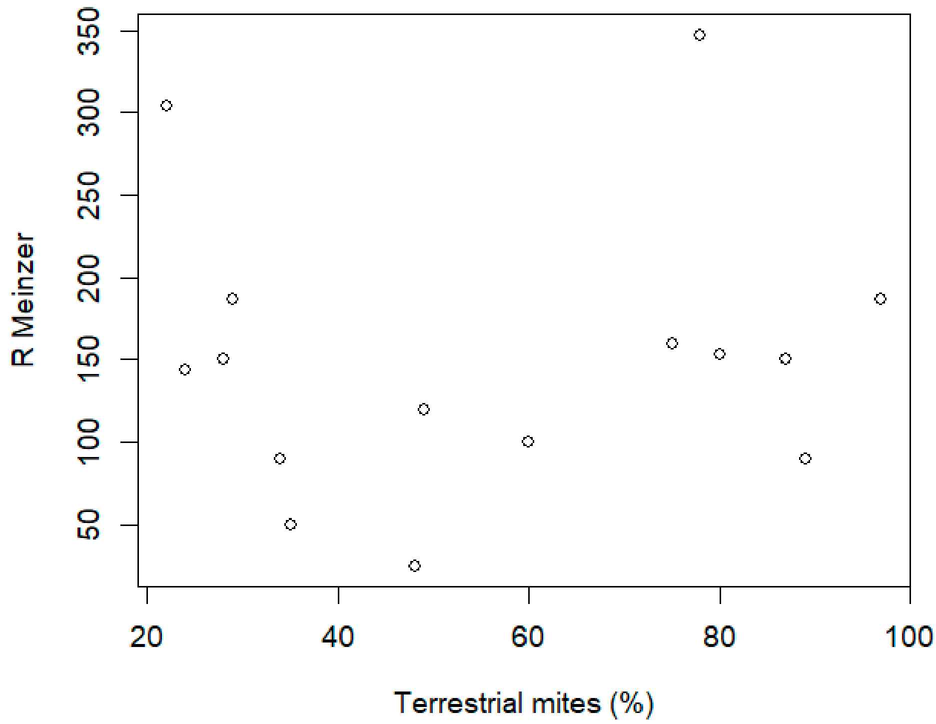


Figure 10. Scatterplot between the percentage of terrestrial mites and the Meinzer index for discharge. The correlation between the two variables was not significant (*r* Pearson = 0.079, *p* = 0.778).

3.7. Faunal (Invertebrate) Desiccation Parameters to Validate Indicators/Metrics Based on Diatoms

Springs with a high desiccation tendency show a high percentage of terrestrial mites, whilst a low percentage of terrestrial mites corresponds to a low desiccation risk (Figure 11).

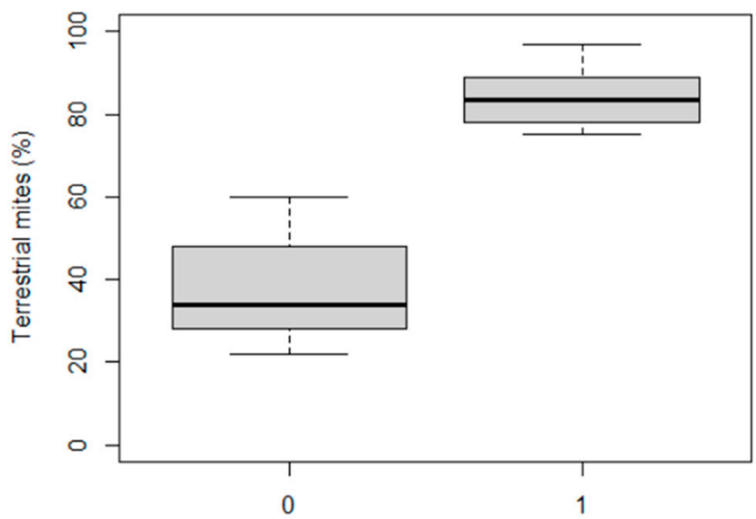


Figure 11. Boxplots of the terrestrial mites (%) in springs with (1) and without (0) tendency to desiccation (intermittent vs. perennial). Median (bold line), first and third quartile (box), and minimum and maximum (whisker) are shown.

The three faunal parameters used to validate the possibility to use diatom-based indicators and metrics are listed in Table 3.

Table 3. Percentage of terrestrial mites, zoobenthos richness, and associated dummy variable for desiccation tendency.

Spring Code	% Terrestrial Mites	% Zoobenthos Species	Desiccation Probability
R-891	28	54	0
R-892	22	60	0
R-893	29	41	0
R-894	48	47	0
R-895	35	58	0
R-896	87	54	1
R-897	34	39	0
R-898	89	38	1
R-899	24	51	0
SW-900	97	47	1
SW-901	49	60	0
SW-902	80	54	1
SW-903	78	55	1
SW-904	75	51	1
SW-905	60	54	0

3.8. Diatom Species Richness in Stable and Fluctuating Discharge Springs

The diatom richness in springs with a high tendency to desiccation is lower than in perennial springs (Figure 12).

3.9. Diatom Red List Species in Stable and Fluctuating Discharge Springs

No significant difference in the percentage of threatened Red List species in the two spring groups (0 and 1) has been found (Figure 13).

3.10. Aerial Diatom Species in Stable and Fluctuating Discharge Springs

A slightly higher but not significant percentage of aerophilic species in springs with a high tendency to desiccation has been found (Figure 14).

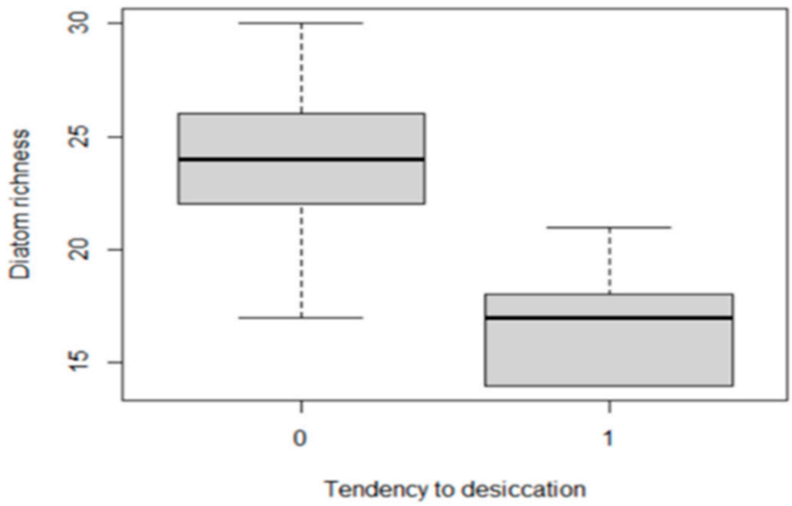


Figure 12. Boxplots of the diatom richness in springs with (1) and without (0) tendency to desiccation (intermittent vs. perennial). Perennial springs (0) have higher species richness than intermittent springs (median was 24 vs. 17). *t* test = 3.89, $p = 0.002$). Median (bold line), first and third quartile (box), and minimum and maximum (whisker) are shown.

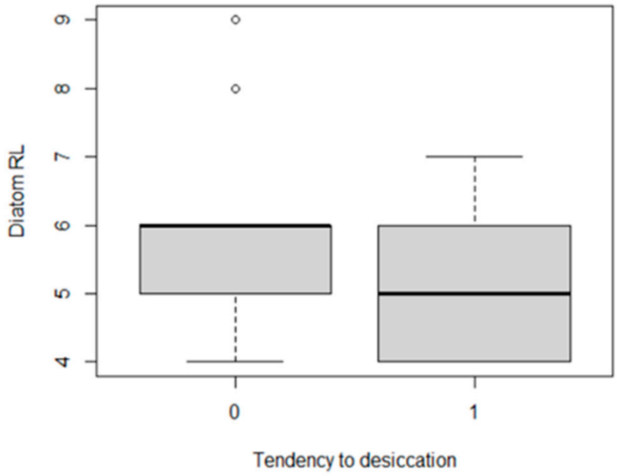


Figure 13. Boxplots of the RL diatoms in springs with (1) and without (0) tendency to desiccation (intermittent vs. perennial). Difference was not significant (the two boxes overlap). Median (bold line), first and third quartile (box), and minimum and maximum (whisker) are shown.

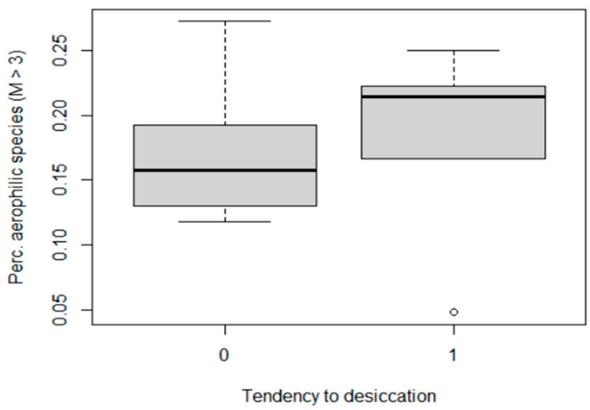


Figure 14. Boxplots of the percentage of aerophilic diatom species (with moisture value > 3) found in springs with (1) and without (0) tendency to desiccation (intermittent vs. perennial). Difference was not significant (the two boxes overlap). Median (bold line), first and third quartile (box), and minimum and maximum (whisker) are shown.

3.11. Ecological Guilds in Stable and Fluctuating Discharge Springs

A *X*-squared test was performed to find a relationship between the ecological guilds and desiccation tendency; data show no association (Table 4).

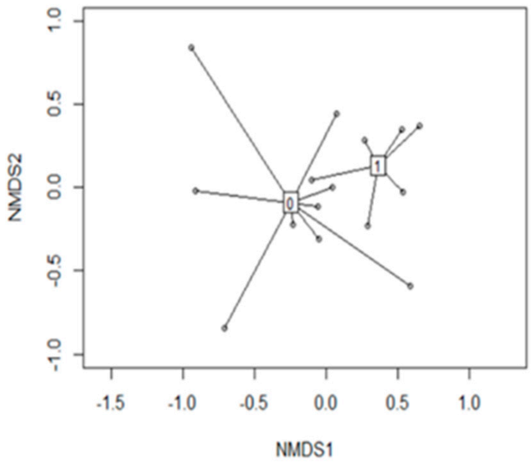
Table 4. Ecological guilds in stable and fluctuating discharge springs. *X*-squared = 0.22198, *df* = 1, *p*-value = 0.6375. No association has been found.

		Spring Type 0 (Perennial)	Type 1	Total
High profile (N of sp)	Observed	7	9	16
	Expected	10.8	5.2	
Low profile	Observed	84	40	124
	Expected	83.4	40.6	
Motile	Observed	71	30	101
	Expected	69.9	33.1	
Total Observed		162	79	241

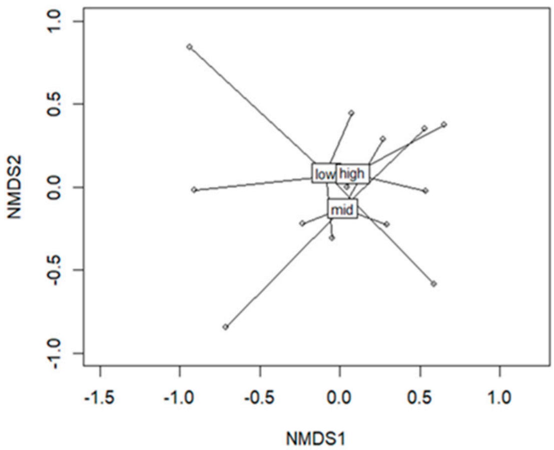
3.12. Diatom Communities in Stable and Fluctuating Discharge Springs

A NMDS ordination of the diatom samples shows that springs with a high or low tendency to desiccation have typical and distinct diatom assemblages (Figure 15a), and

NMDS re-ordination of the three Meinzer variability classes (low, mid, high) shows that diatom assemblages do not separate according to discharge variability: For these diatom groups, it is important if springs get dry or not but not so much if there are marked discharge fluctuations (Figure 15b).



(**a**)



(**b**)

Figure 15. (**a**) Ordination of the diatom samples using NMDS and showing the springs with (1) or without (0) tendency to desiccation (intermittent vs. perennial). Different diatom assemblages were found in the different hydrological types (F = 2.51, $p = 0.008$). (**b**) Ordination of the diatom samples using NMDS and showing the springs with low, mid, and high Meinzer index (see MM for further information). Differences were not significant (F = 0.53, $p = 0.989$).

3.13. Diatom Indicators in Stable and Fluctuating Discharge Springs

Indicator species values (IndVal) analysis was performed in order to find indicator species in the two spring groups (0 and 1). It combines a species' relative abundance with its occurrence in the group of sites used for analysis [41].

The IndVal method identified *Amphora pediculus* as indicator of springs with low desiccation tendency ($p = 0.033$), and *Eunotia minor* ($p = 0.048$), *Meridion circulare* ($p = 0.056$), *Planothidium lanceolatum* ($p = 0.017$) and *Sellaphora nigri* ($p = 0.006$) as indicators of springs with high desiccation tendency.

4. Discussion

We could identify several metrics using diatoms, and it has been possible to validate them using physical parameters (such as the Meinzer index), and also using faunal data (such as the percentage of terrestrial mites). Through this study, it was possible to provide the first information regarding diatom communities living in the springs of Steigerwald Nature Park and of the UNESCO Rhön Biosphere Reserve and their ecological characteristics.

The abiotic parameters measured confirmed that the springs studied have a wide range of conductivity, and we could thus study the diatom communities along a relatively wide conductivity gradient.

In Steigerwald and only in this protected area, it was observed that the average conductivity values vary from very low to very high. The lowest values were recorded in three springs (SW904, SW903, SW900) that were also affected by total desiccation, probably because they are subject to disturbance and variations in flow. It could be assumed that the three springs are placed on a geological formation that determines the development of small surface aquifers that make the springs that they feed more subject to desiccation.

Nitrate values were higher than expected for oligotrophic springs. Chemical analyses showed a wide range including also high values. Nevertheless, in both protected areas, species with autotrophic metabolism with respect to nitrogen (Van Dam index N mostly = 1 or 2) were found. High values were recorded also for phosphate, but the two ions do not increase in concentration in concomitance. The information regarding possible activities, anthropic impact, and grazing for the surrounding areas is few. Therefore, further investigations are needed to correctly understand this unexpected result. In spite of the fact that these are protected areas, it could be that there is some intensive agriculture, grazing, and forestry and the presence of game. High nitrate concentrations were observed, for instance, in D900 which has a nearby road and agricultural land; D901 is characterized by the presence of a path; D905 feeds a nearby pond and is surrounded by an uncultivated lawn.

All the springs studied have an average pH around neutrality and most of the species are alkaliphilous or circumneutral according to Van Dam et al. [14].

The average flow rate is very low for all the study sites with discharge < 1 L/s: These are very small springs, and the occurrence of aerial, xerophilous, and first-colonizer species reflects that these habitats are affected by pronounced flow intermittence. Climate change, increasingly frequent droughts, and less frequent rainfall could cause these springs to become temporary with occasional complete desiccation events.

The diatom assemblages of the UNESCO Rhön Biosphere Reserve and of Steigerwald Nature Park share several species, confirming that the communities of the two protected areas are not so different from each other, as confirmed by multivariate analysis of variance (Figure 6). These are widely distributed species, and only *Achnanthidium dolomiticum* and *Humidophila perpusilla* seem to be very competitive in environments with intermittent water flow. Even the Rhön species with a moisture index M = 5 (present almost exclusively outside the water) are the same as those found in Steigerwald (*Adlafia bryophila*, *Humidophila perpusilla*).

The percentages of species with moisture indices M = 4 or 5 (=not linked to the submerged environment) were lower than those found by Angeli et al. [42] for the two groups of springs they studied (CESSPA 18% and CRENODAT 20%). As for other analyzed parameters, this metric may be relatively low due to the moderate extent and relative geological uniformity of the areas studied here, but the similarity of diatom data in the two areas reflects similar flow rates, as confirmed by abiotic parameters.

The cumulative percentage of species belonging to the threatened, possibly threatened, and rare categories of the Red List is a metric sensitive to the increase of nitrate concentrations and to the alterations of the natural spring morphology with water abstraction [13]. The percentages of 41% for Rhön and 42% for Steigerwald are higher than that found in the 25 springs of the CESSPA project (24%) located in the Adige basin in the area of Verona and with high nitrate concentrations but lower than that found in 32 springs of the CRENODAT project (47%) located in different mountain basins and in the Autonomous Province of Trento and with much lower nitrate concentrations [42]. In the present study, although nitrate concentrations were high for oligotrophic environments, the percentage of threatened species was nevertheless significant, reinforcing the hypothesis that discharge variability is the prevalent environmental determinant in this case. However, the percentage of diatom species in threat categories of the Red List is lower than values (around 50%) reported by Cantonati et al. [13] for Alpine springs and lakes and for the Berchtesgaden National Park in Germany, all ecosystems with high ecological integrity. This could be due to the partial influence of the high nitrate concentrations on this metric [13,43].

The variability represented by the dispersion (distance) of the points around the centroids (Figure 15a) is greater in the springs with a low desiccation probability than in those with a high tendency to dry out. This could be explained by the fact that the assemblages of springs "1", with a high desiccation tendency, consist of a lower number of more specialized and more selected species due to the effect of the stress factor. The species that inhabit the more stable "0" sources can be more diversified according to lithology, pH, shading, etc. Furthermore, this analysis showed that there are typical assemblages in each group: The intermittent springs have characteristic communities that differ from those found in perennial springs.

The increased richness in perennial springs shown in the box plot (Figure 12) suggests that the disturbance created by desiccation events can affect population dynamics, for instance, by reducing the number of species in that community.

A comparison between the number of endangered species belonging to the Red List in perennial springs (0) and intermittent ones (1) showed that there is no significant difference (Figure 13). This could be because diatom studies of unstable waters were much rarer than those on permanent waters and large rivers. In addition, many species of non-perennial springs are less known in their distribution and ecology and therefore can be found on the Red List with a degree of threat that does not necessarily correspond to their real condition: Further investigation of these environments should clarify these data.

Unlike in the springs of the Alps where the species richness was often found to be higher on bryophytes as compared to the lithic material, in the present study, the Shannon–Wiener diversity index and species richness on the two substrata (lithic and plant material) were similar (Figure 7). Bryophytes offer a set of micro-niches to diatoms in springs, and this is not found on stones [5,44]. Probably, since the sampling sites of the present study were all strongly shaded and with poorly developed bryophyte vegetation, they may not have the marked microhabitats found in more structured springs of the Alps. Therefore, the lower complexity would be reflected in the similar diversity and species richness on the two substrata. This is also in good agreement with Taxböck et al. [45] who, in their study about elevation gradients in Switzerland, found that diatom species richness in spring ecosystems increases with the complexity of the habitat (and the altitude).

The lack of relationship between the percentage of terrestrial mites and the Meinzer index for discharge suggests that, for these organisms, the key point is complete desiccation and not marked discharge fluctuations (Figure 10).

In the present study, we also investigated possible associations between the Passy diatom ecological guilds [46] and the tendency to desiccation. No significant correlation could be found. The lack of association may be due to the fact that the number of samples considered remains limited to uncover this type of correlation.

The Meinzer index for discharge has been re-arranged into three classes, "low", "mid", and" high", and has been related to diatom assemblages (Figure 15b). We found that the diatom communities do not separate according to flow variability. As already noted for terrestrial mites, complete desiccation events seem to be the discriminant factor.

The IndVal confirmed *Eunotia minor* and *Meridion circulare*, the tolerance desiccation of which was already known, as possible indicators for intermittent springs.

The significant relationship between mean flow rate and desiccation tendency obtained from faunal (invertebrate) data (Figure 9) supports our approach to use a completely different group of organisms to validate diatom preferences and metrics. This "faunal" approach might suggest desiccation tolerance for diatom species not supposed to possess this trait before.

5. Conclusions

In spite of the limited number of springs that could be sampled in this study, our results could contribute important points to the use of diatoms as indicators of discharge variability/desiccation:

- It is possible to use diatoms in spring ecosystems as indicators of water level / discharge fluctuations and desiccation probability.
- Springs affected by discharge variability/desiccation have lower diatom species richness.
- Springs affected by discharge variability/desiccation have distinct diatom communities.
- Diatom indicators and metrics can be validated using other components of the spring biota, namely invertebrates.
- Larger databases will be necessary to identify the most suitable diatom indicators of discharge variability/desiccation in springs.

Author Contributions: Conceptualization, M.C. and D.S.; methodology, M.C., R.G. and S.S.; validation, M.C., R.G., S.S. and G.M.; formal analysis, D.S.; investigation, R.G., A.S., S.V. and C.C.; data curation, D.S., C.C., M.C., R.G., A.S., S.V., S.S. and O.P.B.; writing—original draft preparation, C.C., M.C. and O.P.B.; writing—review and editing, M.C., G.M., C.C., A.S., S.V., A.A.S., S.S., O.P.B., D.S. and R.G.; visualization, D.S., S.S., C.C., A.A.S., O.P.B. and M.C.; supervision, M.C. and G.M.; project administration, A.S. and S.V.; funding acquisition, R.G., A.S. and S.V. All authors have read and agreed to the published version of the manuscript.

Funding: For monitoring, the UNESCO Rhön Biosphere Reserve received funding from the Bavarian State Ministry of Environment and Consumer Protection, the Hessian Ministry of Environment, Climate Protection, Agriculture, and Consumer Protection, and the Thuringian Ministry of Environment, Energy, and Nature Conservation. The sampling in Steigerwald was part of the project "*Lebensraum Quelle—Monitoring im Klimawandel*".

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data are available upon request from the authors and from the UNESCO Rhön Biosphere Reserve Administration and the Bavarian Environment Agency.

Acknowledgments: We thank the Bavarian State Forest Enterprise (*Bayerische Staatsforsten*) and the forest administration of Ebrach for providing the monitoring sites in Steigerwald. For providing the monitoring sites in the Rhön Biosphere Reserve, we thank the Bavarian State Forest Enterprise (*Bayerische Staatsforsten*), the forest administration of Bad Königshofen, the forest administration of Thuringia (*Thüringen Forst*), the forest administration of Hesse (*Hessen-Forst*), the community of Bischofsheim in der Rhön, and private persons from the community of Kaltennordheim and Bischofsheim in der Rhön. The sampling of diatoms was permitted by the higher nature conservation authorities of lower and upper Franconia and the higher nature conservation authorities of Hesse and Thuringia. We also thank the rangers of the Rhön Biosphere Reserve for their support with the field work.

Conflicts of Interest: The authors declare no conflict of interest.

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