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A Simple and Inexpensive Method to Gain Diatom Absolute Abundances from Permanent Mounts in Hydrobiological and Paleoecological Research

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Abstract: Here, we describe and discuss a method based on microscopical field of view (FOV) area to estimate diatom absolute abundances (densities or concentrations), and we statistically verify its reliability, also comparing it to another commonly used method (microspheres). To test the new method, we purposely performed replicate counts, both with the FOV and with the microsphere method, on both subfossil and recent material (samples) from mires. Intraclass correlation (ICC) revealed a high degree of agreement between the measurements obtained for the replicates with each of the two methods, suggesting that both are reliable for measuring diatom valve concentrations. However, the FOV consistently overestimated diatom absolute abundances, as compared to the microsphere method, and the ICC value used to assess the reliability of the two methods combined suggested that the two methods cannot be used interchangeably. The FOV method is relatively simple, has a lower cost, wider applicability, higher resolution, and warrants compatibility with existing datasets. However, there may also be drawbacks, such as the potential for sample distortion during the concentration process. Therefore, it is important to carefully evaluate the strengths and limitations of the different methods before adopting one for specific research or applied questions.

Keywords: diatoms; absolute abundances; methodology; mires; microspheres; FOV

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1. Introduction

Diatoms (Bacillariophyta) are unicellular, often colonial, microalgae, the cell walls of which—known as frustules (each including two valves)—are composed of amorphous silica, which, if conditions are suitable, can be preserved indefinitely in the sediments [1]. The valve face of each theca is intricately patterned, allowing it to be identified to the species level [2].

For microscopic examination, cells are usually cleaned to remove their organic contents and allow details of the siliceous component of the frustule to be revealed [2]. The routine analysis of diatoms only implies the expression of the results as relative frequencies. Percentage abundance refers to the proportion of diatom valves in a sample relative to the total number of all types of count units in the sample. However, knowing the density or concentration of diatom valves provides a more precise measurement of the number of diatoms per unit volume or area in the sample. This information is important for understanding the biomass, productivity, and ecological roles of diatoms in their natural habitats.

Additionally, knowledge of the density or concentration of diatom valves can help to identify changes in their abundance and distribution over time and space, which is important for monitoring and managing aquatic ecosystems. For instance, changes in diatom density or concentration can indicate changes in nutrient availability, water quality, or other environmental factors that may affect the health of aquatic ecosystems.

Different methods have been proposed for the calculation of diatom valve concentration in permanent slides. Battarbee [3] introduced the random settling method based on the principle that a sedimented part of a "well-mixed" suspension achieves random distributions for algal counting. It assumes that an aliquot accurately represents the total sample, and it involves the use of an evaporation tray in which diatom suspensions sediment randomly on slides to create quantitative slides of microfossils after evaporation. However, this method can create a subsampling bias since samples must be extremely clear of dissolved salts, and it requires several washing steps [4], especially when the sample is rich in dissolved ions [5].

As an alternative to the use of suspensions, there are techniques that utilize counting chambers. The Utermöhl method [6] assumes that phytoplankton settle following a Poissonian distribution in the counting chamber and, thus, can be identified and enumerated using an inverted microscope.

Other quantitative analysis involves the use of added markers. The addition of *Lycopodium clavatum* marker-grains as a spike to a sample was widely used in absolute pollen analysis since it was introduced by Stockmarr [7]. This method was tested for diatom analysis [8]. It was established that the addition of a known concentration of spores to the diatom sample offers a frame of reference for comparison with diatom counts. Another "added marker" method was proposed by Battarbee [2]. It involves the addition of a known quantity of divinylbenzene microspheres to a known mass of a sample.

There has been more attention paid to methods able to produce benthic/sediment diatom absolute concentrations among researchers dealing with paleoecology, and especially with marine sediments [9–11].

Overall, the choice of method for calculating diatom valve concentration in permanent slides depends on the available resources, the nature of the sample, and the research question being addressed. The accuracy and precision of diatom valve concentration calculations can be affected by a number of factors, such as sediment mixing and bioturbation, diagenesis, grain size, and sample preservation [12]. Therefore, it is important to use rigorous quality control procedures and to interpret the results in the context of the known limitations and uncertainties of the data. Any reliable test method should produce consistent, accurate, and reproducible results and should be able to detect small differences specific to the property or feature being measured [13]. It should also be robust and traceable to a recognized standard. Thus, any method developed for the calculation of diatom valve concentration should be standardized and reproducible and should be suitable to provide accurate and precise diatom counts. Accordingly, quality control measures should be implemented to ensure that the method is reliable and consistent and that any sources of variability or error are identified and addressed.

Previous papers on methods allowing one to obtain diatom absolute abundances of benthic or paleoecological materials were mostly related to marine paleoecological studies and typically not focused on adapting and promoting such methods in ecological and environmental/applied diatom studies dealing with present-day samples. Therefore, in this work, with the specific objective of demonstrating the utility of the straightforward integration of this relatively simple and inexpensive method in current fundamental and applied diatom research, we aim to assess the reliability of two different methods for the calculation of diatom valve concentrations in permanent slides: microspheres and a here-proposed alternative method based on microscopical fields of view.

2. Materials and Methods

A mire sampling campaign was organized in the Adamello-Brenta Nature Park (south-eastern Alps, Autonomous Province of Trento, Italy), by the Limnology & Phycology Section of the MUSE in Trento in the summer of 2021. Both recent (from surface sediment and bryophytes) and paleolimnological samples were collected.

Six recent samples were selected, three of epipelic diatoms and three of epiphytic diatoms. For surface sediment samples, three squares of 36 cm^2 were sampled for each mire pool. Plastic squares with a central carved area of 3 × 3 cm were used to define the area to be sampled. The sediment was then sampled by drawing a plastic tube across the surface of the sediment, allowing it to fill with a mixture of sediment and water. For each sample, the surface area of sediment taken, therefore, corresponded to 108 cm^2. For the epiphytic diatom samples, submerged bryophytes were collected in three different points for each pool.

Four samples from peat cores were selected. Sampling was conducted using a stainless-steel Belarus corer, providing semi-cylindrical peat sections, which were 50 cm long and 10 cm wide. Once collected, cores were photographed, wrapped in polyethylene cling film, placed in specifically built boxes, brought to the lab, and frozen at −18 °C. Belarus cores were cut while frozen into 3.0 ± 0.3 cm slices using a stainless-steel knife.

Accurate records were kept of the volume of sediment, the fresh weight of bryophytes, or peat used for digestion (oxidation), which were necessary for subsequent quantitative calculations. Fresh bryophyte and peat material were dried for 24 h at 105 °C to obtain their respective dry weights.

All samples were treated with hydrogen peroxide and hydrochloric acid. The digested material was then diluted to a final volume of 15 mL.

The cleaned diatom suspension was thoroughly shaken to ensure homogenization of the solution. An aliquot was taken from the suspension using a microliter pipette and placed on a coverslip. Distilled water was added to obtain the optimal concentration for the slide. To achieve a uniform distribution of diatoms, the slides were allowed to air-dry without the use of a hotplate. Cleaned valves were mounted in Naphrax® (Brunel Microscopes Ltd., Chippenham Wiltshire, UK). Previous studies have suggested that diatoms tend to be more concentrated near the center with less density near the margin [6,14]. To minimize the bias related to the non-uniform distribution of the diatoms, fields of view (FOVs) examined were selected following vertical traverses that covered both more and less concentrated regions. Six permanent mounts were prepared for each sample without microspheres, and three replicates were made for samples with microspheres. A minimum number of 400 valves was identified and counted from each slide under ×1000 magnification using a Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany) and a Leica DME (Leica Microsystems Inc., Wetzlar, Germany) considering the exact number of valves for each species and the number of microscopical fields of view (FOVs) observed. Ideally, there should be 5–15 valves per field of view when viewed at x1000 magnification [15]. Diatom counting was carried out along coverslip transects to prevent any bias in estimates of species composition and density.

The absolute abundance (Dabs) was expressed as the number of valves per gram of dry weight (valves/g dry weight) or valves per cm^2 of surface sediment (valves/cm^2 surface sediment) and was calculated as follows:

$$\text{Valves in coverslip area} = \frac{\text{coverslip area} * \text{valves identified and counted from each slide}}{\text{field of view area} * \text{number of fields of view observed}} \quad (1)$$

$$\text{Valves in the whole sample} = \frac{\text{valves in coverslip area} * \text{final digestion volume}}{\text{volume of digested sample mounted on slide}} \quad (2)$$

The total number of valves in the whole sample is used to calculate valves/g Dry weight or valves/cm^2:

$$\text{Valves per g of dry weight} = \frac{\text{valves in the whole sample}}{\text{dry weight of sample used for digestion}} \quad (3)$$

$$\text{Valves per cm}^2\text{ of sediment} = \frac{\text{valves in the whole sample}}{\text{sample volume of sediment used for digestion}} \quad (4)$$

Diatom taxa were identified according to Cantonati et al. [16], Krammer [17–19], Lange-Bertalot et al. [20–22], and Van de Vijver et al. [23].

Samples contain a notable oligotrophic microflora that is particularly rich in pennate forms of the genera *Cymbopleura*, *Encyonema*, *Eunotia*, *Gomphonema*, *Kobayasiella*, *Nitzschia,* and *Pinnularia.* The centric microflora is represented by the genus *Aulacoseira.* The diatoms mentioned above range in size from 10 µm to >100 µm.

Statistical Analyses

We used intraclass correlation (ICC) [24] as a statistical measure to assess the reliability of our test. The ICC is a measure of how stable over time a test is. ICC is a useful tool for evaluating the reliability of a test, as it takes into account the variability in ratings provided by different analysts or replicates. ICC is a type of correlation coefficient that ranges from 0 to 1. An ICC of 0 indicates no agreement between replicates, while an ICC of 1 indicates perfect agreement. The closer the ICC is to 1, the more reliable the test is considered to be. To calculate ICC, one must collect diatom valve concentration results from at least three replicates across all analyzed samples. The ICC value was calculated based on the variance in the scores produced at each replicate and the variance in the total scores of all the analyzed samples. We calculated the ICC for the diatom valve concentrations obtained with the method proposed in this article but also using the microsphere method. Finally, the ICC was calculated with the results of both methods in common to assess whether there were differences between the results obtained with each of the methods. ICC values were obtained using the R package 'psych' [25]. There are different types of ICC, such as ICC(1), ICC(2), and ICC(3), which are appropriate for different types of reliability assessments. ICC(2) and ICC(3) are typically used for assessing the reliability of tests with multiple replicates.

3. Results

Diatom valve concentration was calculated for 11 samples with replicates. The results ranged from 4.88×10^5 to 2.58×10^{10} valves g^{-1}, with considerable scatter across this range up to values of 7.67×10^7 valves g^{-1} and 6.93×10^9 valves g^{-1} using the microspheres and the FOV method, respectively (Figure 1).

The results of the calculation for concentrations of diatom valves using the two methods, microspheres and FOV, are shown in Table 1, as a function of the mean and relative standard deviation (RSD) obtained for the three replicates analyzed for each sample. RSD, also known as the coefficient of variation, is a statistical measure that expresses the variability in a set of data as a percentage of its mean. We can compare the variability in the different methods used for the concentration values, allowing for a meaningful comparison between both sets of data. On the other hand, the RSD expresses the variability in the data relative to the mean, providing a standardized measure of variability that is independent of the scale of the data.

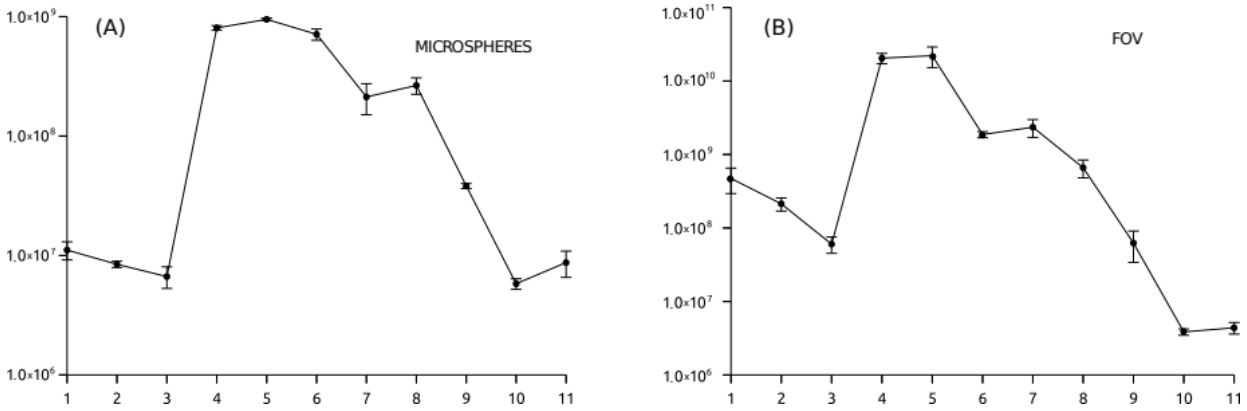

Figure 1. Diatom concentration comparison (axis Y) of replicate slides (axis X) using both the microspheres (**A**) and FOV (**B**) methods. Black dots represent the average valve concentration for each sample while error bars show the data range for the replicate slides. Diatom vale concentration is expressed on a logarithmic scale.

Table 1. Average diatom valve concentrations (valves g^{-1}) and relative standard deviation (RSD) for the samples analyzed using the microspheres method and our proposed method (FOV).

	Microspheres		FOV	
Sample	**Average**	**RSD**	**Average**	**RSD**
smp1	11,099,117	9	473,143,004	21
smp2	8,444,613	3	213,438,866	11
smp3	6,664,874	11	60,366,414	14
smp4	804,252,030	2	20,454,375,290	9
smp5	949,281,872	1	22,133,434,744	16
smp6	711,199,231	5	1,871,473,085	6
smp7	212,539,035	17	2,352,374,624	15
smp8	265,306,395	8	661,645,077	15
smp9	38,235,048	3	62,420,591	26
smp10	5,798,106	5	3,856,625	5
smp11	8,719,384	12	4,388,682	9

The results obtained show that the FOV method consistently provides higher concentration estimates than with the use of microspheres. In any case, the value obtained for the RSD is very similar, indicating that both methods do not differ in the amount of variability in the results. There are several potential reasons why the FOV method might produce higher concentration estimates than the microsphere method, from variations in sample preparation to differences in counting efficiency. The two methods differed in how the samples were prepared. For example, if the fields-of-view method involved sampling a smaller volume of the sample, or if it was more prone to sampling larger or more concentrated areas of the sample, this could result in higher concentration estimates. The two methods may have different counting efficiencies, meaning that they may be more or less likely to detect diatom valves within a given sample. For example, if the fields-of-view method was more likely to detect small or poorly preserved diatom valves, this could result in higher concentration estimates. Furthermore, it is possible that the microsphere and field-of-view methods differed in their instrumental error. For example, if the fields-of-view method was more prone to producing false positives or negatives due to the microscope or other equipment used, this could result in higher concentration estimates. In any case, it is important to carefully evaluate each of these potential sources of error in order to determine the most likely cause of the observed overestimation.

The intraclass correlation coefficient (ICC) was used to assess the reliability of two methods of measuring diatom valve concentration in samples (Table 2). The ICC value obtained for the two methods was very similar, with a value of 0.98 (95% confidence

interval (CI): 0.95–0.99). A high ICC value close to 1 indicates a high degree of agreement between the measurements obtained for the replicates with each method and suggests that both methods are reliable for measuring diatom valve concentration. The ICC value was statistically significant ($p < 0.001$). However, when the ICC value was used to assess the reliability of the two methods combined, it fell to 0.40%. These findings suggest that the two methods might be reliable but cannot be used interchangeably.

Table 2. Intraclass correlation coefficients for the analyzed samples using the microspheres and FOV method and the coefficient for both methods combined. Upper and lower limits for the confidence classes and *p*-values are also shown.

	Interclass Correlation Coefficients		
	FOV	**Microspheres**	**Combined**
ICC2	0.95	0.98	0.40
Lower bound	0.93	0.95	0.17
Upper bound	0.98	0.99	0.71
p-value	<0.001	<0.001	<0.001

However, it is important to note that these results are limited by the small sample size and the potential for error measurement. Further studies with larger sample sizes and more robust methods are needed to confirm these findings and assess the validity of the measurements.

4. Discussion

So far, most approaches to diatom analysis have been based on the calculation of relative frequencies [26]). However, using diatom concentrations instead of relative abundances can improve the accuracy, comparability, and interpretability of diatom data, which can enhance their performance as a biological indicator for environmental reconstructions and water quality assessments [27]).

Diatom concentrations provide more quantitative information on the abundance of diatoms in a sample, which can increase the sensitivity of the analysis [28]. This is particularly important for detecting subtle changes in diatom populations, such as those associated with environmental stressors or changes in water quality.

Diatom concentrations allow for more direct comparisons between samples and across different studies. Relative abundances can be influenced by changes in the total diatom population, which can make it difficult to compare samples with different overall diatom abundances. Concentrations provide a standardized measure of diatom abundance that is not influenced by changes in the total population [29]. Diatom concentrations can be analyzed using a wider range of statistical techniques, which can provide more robust and informative results [30]. Relative abundances may be less suitable for some statistical techniques because they are constrained to a limited range (0–100%).

Diatom concentrations can provide insights into the productivity of a lake or other water body, which is important for understanding its ecological health and functioning. Concentrations can also be used to estimate other environmental variables, such as nutrient concentrations, pH, and temperature, which can provide additional information for environmental reconstructions.

There are several sources of error when counting diatom valve concentrations under a microscope [31]. It is important to select a representative sample size. If the sample size is too small, the results may not be accurate. Similarly, if the sample size is too large, it can be difficult to count all the cells or particles accurately. Additionally, diatom cells vary greatly in size and shape, which can make it difficult to count them accurately. Some cells or particles may be missed or counted multiple times if they are similar in size to others. Some samples can have debris or artifacts that can interfere with accurate counting. Differences in preparation techniques can lead to counting bias and errors in concentration calculations [32]. The person counting can introduce bias if there are preconceived ideas

about what is expected to find or if the analyst is influenced by the results of previous counts [33]. Moreover, different counting techniques can produce different results. Overall, it is important to minimize these sources of error through careful sample preparation, use of appropriate counting techniques, quality control measures, and standardization of laboratory procedures.

Errors related to the sample itself include variability in sample size, variability in cell or particle size, and interference from debris or artifacts [34]. These errors are related to the quality and nature of the sample being analyzed and can be minimized through careful sample preparation and quality control measures. On the other hand, errors external to the sample include observer bias, counting technique, and variability in preparation techniques. These errors are related to the counting process and can be minimized through standardized laboratory procedures, training of personnel, and use of appropriate counting techniques.

By understanding these sources of error and taking steps to minimize them, it is possible to improve the accuracy and reliability of the concentration calculations for cells, microorganisms, or particles under the microscope [35].

High ICC values suggest that both methods are reliable for measuring diatom valve concentration but also indicate a high degree of disagreement between the two methods. When the results from both tests are combined within the same dataset and analyzed using ICC, the high ICC value suggests that the measurements from the two methods are not consistent and interchangeable. Anyway, the high ICC individual values indicate that the results obtained from either method can be trusted to be accurate and consistent.

It is important to note that ICC only assesses the reliability or consistency of measurements and does not provide information on the validity or accuracy of the measurements. Therefore, it is important to also consider other factors, such as the relevance and appropriateness of the methods for the specific measurement and context, and whether the measurements are accurate and representative of the true value of the object being measured.

5. Conclusions

Our newly proposed method for calculating diatom concentrations has some advantages over other approaches:

- A lower cost.
- It is relatively simple and requires only basic laboratory equipment, whereas for the microsphere method, more specialized equipment and materials, that can be more expensive, may be necessary.
- Wider applicability: counts based on FOV can also be used for the analysis of the different diatom life forms in a variety of aquatic systems, including lakes, rivers, and marine environments. This means that researchers can easily adapt it, potentially increasing its applicability.
- Higher resolution: our proposed method allows for the counting of individual diatoms, whereas the other methods rely on the counting of external markers. This means that our method may provide higher-resolution data, particularly for smaller diatom taxa.
- Compatibility with existing datasets: there are many existing slides already available that could potentially be re-analyzed for diatom abundance using our proposed method. This compatibility with existing datasets could save time and resources.

It is important to note, however, that there may also be potential drawbacks to using our proposed method for diatom analysis, such as the potential for sample distortion during the concentration process. Therefore, it is important to carefully evaluate the strengths and limitations of the different methods before adopting one for analysis.

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References

1. Serieyssol, K.; Chatelard, S.; Cubizolle, H. Diatom fossils in mires: A protocol for extraction, preparation and analysis in palaeoenvironmental studies. *Mires Peat* **2010**, *7*, 1–11.
2. Battarbee, R.; Jones, V.; Flower, R.; Cameron, N.; Bennion, H.; Carvalho, L.; Juggins, S. Tracking environmental change using lake sediments. In *Terrestrial, Algal, and Siliceous Indicators*; Smol, J.P., Birks, H.J.B., Last, W.M., Eds.; Springer: Dordrecht, The Netherlands, 2001; Volume 3, pp. 155–202.
3. Battarbee, R.W. A new method for the estimation of absolute microfossil numbers, with reference especially to diatoms. *Limnol. Oceanogr.* **1973**, *18*, 647–653. [CrossRef]
4. Battarbee, R.W. Diatom analysis. In *Handbook of Holocene Palaeoecology and Palaeohydrology*; Berglund, B., Ed.; Wiley: New York, NY, USA, 1986; pp. 527–569.
5. Scherer, R.P. A new method for the determination of absolute abundance of diatoms and other silt-sized sedimentary particles. *J. Paleolimnol.* **1994**, *12*, 171–179. [CrossRef]
6. Utermöhl, H. Zur Vervollkommung der quantitativen Phytoplankton-Methodik. *Mitt. Int. Ver. Ther. Angew. Limnol.* **1958**, *9*, 1–38.
7. Stockmarr, J. Tablets with spores used in absolute pollen analysis. *Pollen Spores* **1971**, *13*, 615–621.
8. Kaland, P.E.; Stabell, B. Methods for absolute diatom frequency analysis and combined diatom and pollen analysis in sediments. *Nord. J. Bot.* **1981**, *1*, 697–700. [CrossRef]
9. Bodén, P. Reproducibility in the Random Settling Method for Quantitative Diatom Analysis. *Micropaleontology* **1991**, *37*, 313–319. [CrossRef]
10. Wolfe, A.P. On diatom concentrations in lake sediments: Results from an inter-laboratory comparison and other tests performed on a uniform sample. *J. Paleolimnol.* **1997**, *18*, 261–268. [CrossRef]
11. Esper, O.; Gersonde, R.; Kadagies, N. Diatom distribution in southeastern Pacific surface sediments and their relationship to modern environmental variables. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **2010**, *287*, 1–27. [CrossRef]
12. Smol, J.P.; Stoermer, E.F. *The Diatoms: Applications for the Environmental and Earth Sciences*, 2nd ed.; Cambridge University Press: Cambridge, UK, 2010; p. 667.
13. Bruton, A.; Conway, J.H.; Holgate, S.T. Reliability: What is it, and how is it measured? *Physiotherapy* **2000**, *86*, 94–99. [CrossRef]
14. Alverson, K.D.; Bradley, R.S.; Pedersen, T.F. *Paleoclimate, Global Change and the Future*; Springer: Berlin/Heidelberg, Germany, 2003; p. 220.
15. Taylor, J.C.; Harding, W.R.; Archibald, C.G.M. *A Methods Manual for the Collection, Preparation and Analysis of Diatom Samples*; Version, 1.0.; WRC Report No. TT 281/07; Water Research Commission: Pretoria, South Africa, 2007; p. 60.
16. Cantonati, M.; Van de Vijver, B.; Lange-Bertalot, H. *Microfissurata* gen. nov. (Bacillariophyta), a new diatom genus from dystrophic and intermittently wet terrestrial habitats. *J. Phycol.* **2009**, *45*, 732–741. [CrossRef] [PubMed]
17. Krammer, K. The Genus *Pinnularia*. In *Diatoms of Europe*; Lange-Bertalot, H., Ed.; A.R.G. Gantner Verlag. Kommanditgesellschaft: Ruggell, Liechtenstein, 2000; Volume 1, pp. 1–703. [CrossRef]
18. Krammer, K. Cymbella. In *Diatoms of Europe*; Lange-Bertalot, H., Ed.; A.R.G. Gantner Verlag. Kommanditgesellschaft: Ruggell, Liechtenstein, 2002; Volume 3, pp. 1–584.
19. Krammer, K. Cymbopleura, Delicata, Navicymbula, Gomphocymbellopsis, Afrocymbella. In *Diatoms of Europe*; Lange-Bertalot, H., Ed.; A.R.G. Gantner Verlag. Kommanditgesellschaft: Ruggell, Liechtenstein, 2003; Volume 4, pp. 1–530.
20. Lange-Bertalot, H.; Metzeltin, D. Indicators of Oligotrophy: 800 taxa representative of three ecologically distinct lake types: Carbonate buffered, oligodystrophic, weakly buffered soft water. In *Iconographia Diatomologica*; Koeltz Scientific Books: Königstein, Germany, 1996; Volume 2, pp. 1–390.
21. Lange-Bertalot, H.; Bąk, M.; Witkowski, A.; Tagliaventi, N. Eunotia and some related genera. In *Diatoms of Europe*; Lange-Bertalot, H., Ed.; A.R.G. Gantner Verlag. Kommanditgesellschaft: Ruggell, Liechtenstein, 2011; Volume 6, pp. 1–755.

22. Lange-Bertalot, H.; Hofmann, G.; Werum, M.; Cantonati, M. *Freshwater Benthic Diatoms of Central Europe: Over 800 Common Species Used in Ecological Assessment*; Koeltz Botanical Books: Schmitten, Oberreifenberg, Germany, 2017; p. 942.
23. Van de Vijver, B.; Ector, L.; Beltrami, M.E.; de Haan, M.; Falasco, E.; Hlúbiková, D.; Jarlman, A.; Kelly, M.; Novais, M.A.; Wojtal, A.Z. A critical analysis of the type material of *Achnanthidium lineare* W. Sm. (Bacillariophyceae). *Algol Stud.* **2011**, *136*, 167–191. [CrossRef]
24. Koch, G.G. Intraclass correlation coefficient. In *Encyclopedia of Statistical Sciences*; Kotz, S., Johnson, N.L., Eds.; John Wiley & Sons: New York, NY, USA, 1982; Volume 4, pp. 213–217.
25. Revelle, W. Psych: Procedures for Psychological, Psychometric, and Personality Research. R Package Version 2.2.9. 2022. Available online: https://CRAN.R-project.org/package=psych (accessed on 14 March 2023).
26. Moheimani, N.R.; Borowitzka, M.A.; Isdepsky, A.; Sing, S.F. Standard methods for measuring growth of algae and their composition. In *Algae for Biofuels and Energy*; Borowitzka, M., Moheimani, N., Eds.; Springer: Dordrecht, The Netherlands, 2012; Volume 5, pp. 265–284.
27. Nohe, A.; Goffin, A.; Tyberghein, L.; Lagring, R.; De Cauwer, K.; Vyverman, W.; Sabbe, K. Marked changes in diatom and dinoflagellate biomass, composition and seasonality in the Belgian Part of the North Sea between the 1970s and 2000s. *Sci. Total Environ.* **2020**, *716*, 1–14. [CrossRef] [PubMed]
28. Lavoie, I.; Hamilton, P.B.; Poulin, M. Phytoplankton community metrics based on absolute and relative abundance and biomass: Implications for multivariate analyses. *J. Appl. Phycol.* **2011**, *23*, 735–743. [CrossRef]
29. Bellinger, E.G.; Sigee, D.C. *Freshwater Algae: Identification, Enumeration and Use as Bioindicators*, 2nd ed.; Wiley-Blackwell: Hoboken, NJ, USA, 2015; p. 304.
30. Salkind, N.J. *Encyclopedia of Research Design*; SAGE Publications: Washington, DC, USA, 2010; Volume 1, p. 1776.
31. Alverson, A.J.; Manoylov, K.M.; Stevenson, R.J. Laboratory sources of error for algal community attributes during sample preparation and counting. *J. Appl. Phycol.* **2003**, *15*, 357–369. [CrossRef]
32. Blanco, S.; Álvarez, I.; Cejudo, C. A test on different aspects of diatom processing techniques. *J. Appl. Phycol.* **2008**, *20*, 445–450. [CrossRef]
33. Werner, P.; Adler, S.; Dreßler, M. Effects of counting variances on water quality assessments: Implications from four benthic diatom samples, each counted by 40 diatomists. *J. Appl. Phycol.* **2016**, *28*, 2287–2297. [CrossRef]
34. Kahlert, M.; Kelly, M.; Albert, R.L.; Almeida, S.F.; Bešta, T.; Blanco, S.; Vogel, A. Identification versus counting protocols as sources of uncertainty in diatom-based ecological status assessments. *Hydrobiologia* **2012**, *695*, 109–124. [CrossRef]
35. Kelly, M.; Lewis, A. Assessing the quality of water quality assessments: An analytical quality control protocol for benthic diatoms. *Freshw. Forum* **1996**, *7*, 23–32.