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Diversity and abundance of planktonic ciliated protists: How the analyzer's eye can affect the results

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ABSTRACT. Planktonic freshwater organisms, such as ciliates, play an important role in the transfer of matter and energy and, therefore, in the functioning of aquatic ecosystems. Due to their small size, the analysis of these communities is usually carried out using optical microscopy and various techniques. However, there are many challenges regarding this group, such as the difficulty in identifying them and the lack of experienced personnel for counting these organisms. Therefore, the aim of this study was to compare possible discrepancies in the results of organism counts, taking into account that these results may be influenced by the individual carrying out the sampling. To this end, the differences in the analyses of richness and abundance performed by four (4) analyzers and their respective ratings of the fixed samples were compared. The hypothesis was that more experienced analyzers would record higher values of species richness and density scores than those with less experience. The results obtained were consistent with the initial hypotheses and showed that two of the more experienced analyzers were associated with higher values for the attributes analyzed.

Keywords: plankton; protist; ecology; sampling variability.

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Introduction

Planktonic organisms represent a fundamental component of aquatic ecosystems, among which ciliated protists play a significant role in the transfer of matter and energy within aquatic environments (Oliveira et al., 2024). These microorganisms exhibit high population growth rates, present substantial consumption of phytoplanktonic and bacterioplanktonic production, and serve as an important resource for higher trophic levels in the pelagic food web (Progênio et al., 2024).

Ecological studies on ciliated protists face challenges due to their small size (Elmoor-Loureiro et al., 2023), some of them shared with studies on other groups of microorganisms, whether prokaryotic or eukaryotic. Several techniques, such as counting in Ütermohl chambers using fixed samples, have been employed, displaying advantages and limitations. The appropriate selection of these methods, considering the ciliate group and research objectives, is essential for success (Shah & Ahmed, 2024). For instance, the use of the Ütermohl chamber allows high-magnification counts under an inverted microscope via cell sedimentation and enables analysis in projects involving a large number of samples. However, sedimentation time, the need to fix organisms and debris in the sample can hinder analysis (Freibott et al., 2014).

Additionally, beyond the intrinsic difficulties of ciliate-specific techniques, the lack of trained specialists for identifying them poses a challenge to research development in this field. This often leads to observation errors and misidentifications of ciliated protists, typically due to the observer's lack of experience (Foissner, 1994; Regali-Seleghim et al., 2011). Training in the identification and counting of ciliate protists requires both morphological knowledge and practical experience with microscopic techniques. Direct microscopy by a trained experimenter provides accurate abundance measurements for single-species or complex communities and is unrivalled in terms of registering specific qualitative behaviors and morphology for species identification (Altermatt et al., 2015).

Regarding the need for experienced analysts in protist biodiversity surveys, Culverhouse et al. (2003) demonstrated that less experienced analysts struggle to categorize specimens from species with significant morphological variation. In the same study, the authors noted that experts (who routinely engage in species analysis) are expected to achieve identification accuracy rates ranging from 84 to 95%. Thus, this study aimed to evaluate the impact of analyzer experience on the results of identifying and counting planktonic ciliates, particularly considering the methodological challenges associated with the small size of these organisms.

The central hypothesis was that differences in community attributes between observers with greater or lesser experience would be significant (where the analyzers with greater experience would record a higher number of species and densities), as all observers had some level of expertise in group diversity, microscope use, and analysis protocols. Methodologically, the study compared the richness and density of planktonic ciliates recorded by four observers: two more experienced (A1 and A2) and two less experienced (A3 and A4) in analyzing fixed samples.

Material and methods

In order to conduct the experiment, 40 liters of water were collected from a fishing pond in the city of Maringá, state of Paraná, Brazil. After two days of acclimation in an aquarium to stabilize the environment, the water was homogenized and three 2-liter samples were taken as analytical replicates. The water from each replicate was concentrated to 100 mL by filtration through a 10-micrometer plankton net and fixed using the Sherr & Sherr (1993) methodology, which combines alkaline Lugol solution, formaldehyde buffered with calcium borate, and sodium thiosulfate. To facilitate cell observation, the samples were stained with Rose Bengal dye and quantified using the Utermöhl methodology. Analyses were performed in 3 mL sedimentation chambers under an inverted microscope, with the chamber analyzed in full, and organisms identified to the lowest possible taxonomic level. In total, 12 samples were obtained, considering the three replicates, each one analyzed by the four observers.

The four observers were classified into two experience groups based on their training time and practice in identifying ciliate protists. The more experienced observers (A1 and A2) had over 4 years of formal training in ciliate taxonomy and counting, including supervised sample analysis. The less experienced observers (A3 and A4) had approximately 1 year of training, with limited exposure to different taxonomic groups and less independent practice.

The existence of significant differences in the results obtained by the different observers was tested using an unpaired t-test. To compare whether differences existed between individual observers, a paired t-test was performed. Additionally, we compared the impact of including less experienced observers (here, we considered the results obtained by 'All Analyzers') on the outcomes that would have been achieved had only the more experienced observers participated in the analyses. Statistical analyses and graphs were executed using the R Core Team (2024) software and the RStudio platform, employing the vegan and ggplot2 packages.

Results

The results obtained by all analyzers revealed the identification of 26 species across five orders, with Hymenostomata and Oligotrichida being the most species-rich, each represented by eight species. A paired t-test demonstrated significant differences between the analyzers for both community richness and density ($p < 0.001$; Figures 1A and 1B). The comparison between individual analyzers revealed significant differences in density between A1 and A4 ($t = 5.572$, $df = 2$, $p = 0.030$), in both richness and density between A2 and A3 ($t = 10.390$, $df = 2$, $p = 0.009$ and $t = 8.022$, $df = 2$, $p = 0.015$, respectively), and marginally significant differences in richness ($t = 3.211$, $df = 2$, $p = 0.084$) and significant differences in density ($t = -2.218$, $df = 2$, $p = 0.014$) between A2 and A4.

Regarding the impact of including all analyzers on the results obtained by the more experienced analyzers, some differences were observed for species richness and density (Figure 1C and 1D). For 'All Analyzers,' as expected, greater variability in the data was observed, particularly for density, indicating that the inclusion of less experienced analyzers can introduce substantial variability to the results. To further assess the impact of less experienced analyzers, we evaluated the percentage differences in the mean results compared to those obtained if only the more experienced analyzers had participated. These results indicated an average loss of 15.78% in species richness and a 34.57% loss in the total density of planktonic ciliates.

Considering the observed loss in both richness and density, we sought to determine whether this loss was specifically associated with any particular taxonomic group of recorded ciliates. Clear differences were observed across the different taxonomic groups regarding community attributes among the analyzers, with certain taxonomic groups being underrepresented in the results obtained by specific analyzers. For instance, individuals from the order Gymnostomatida were observed exclusively by some analyzers, and a reduction in both richness and density of Scuticociliates was noted among the less experienced analyzers (Figure 2).

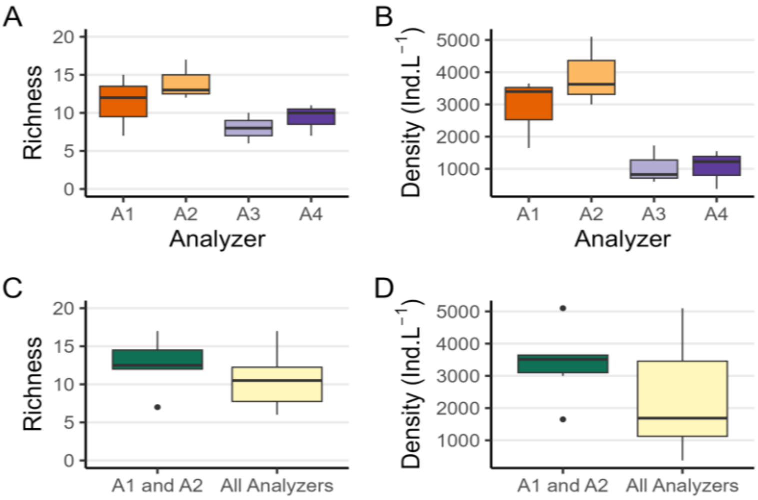

Figure 1. Variation of the richness (A) and density (B) of the ciliate community between different Analyzers. Comparison of richness (C) and density (D) of planktonic ciliates between the most experienced analyzers (A1 and A2) and the group composed of all analyzers (All Analyzers). A = Analyzers (one to four). The central line in graphics denotes the median values.

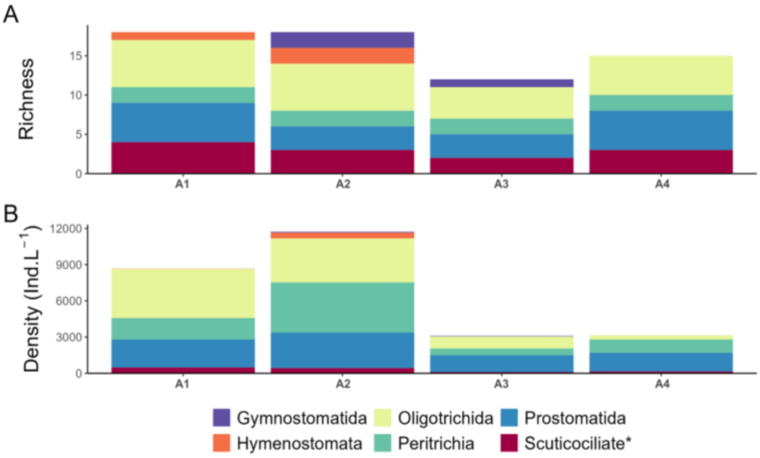

Figure 2. Contribution of taxonomic groups to the richness (A) and density (B) of ciliated protists between analyzers. Scuticociliate* is part of the order Hymenostomata, but in this study, this group was separated in order to observe the variation of these smaller protists between the analyzers. A = Analyzers (one to four).

Discussion

The more experienced analyzers (A1 and A2) recorded higher species richness and density compared to the less experienced ones (A3 and A4). Although all analyzers have practice in ciliated protist analysis, the precision of observations may vary due to biases associated with the analyzers, often related to 'human error' (Elphick, 2008). These errors include mistakes in organism identification and counting failures, such as duplication or underestimation of individual numbers (Elphick, 2008), and possibly underestimation of species richness. This discrepancy suggests that some cells are observed by certain analyzers but not by others, even though this experiment employed staining techniques to facilitate organism visualization.

In studies of planktonic ciliated protists, specific biases can influence the counting process and exacerbate human error, thereby affecting results. For example, the difficulty in visualizing and identifying small species, such as Scuticoliates from the order Hymenostomata, can significantly impact outcomes (Li et al., 2024). This challenge appears to have been the primary driver of reduced richness and density in this study when comparing more and less experienced analyzers. Conversely, the Oligotrichida group of protists has pronounced body and ciliary features (Liu et al., 2019) and is typically present in high densities in the plankton of freshwater environments, making them easier to detect and identify in samples (Grattepanche et al., 2014).

Regarding the observation of specific ciliate protist orders, the failure to detect organisms from the Gymnostomatida group supports the 'human error' phenomenon discussed by Elphick (2008). Gymnostomatid protists are known to inhabit diverse environments, including anoxic sites (Schwarz & Frenzel, 2003) and mammalian rumen (Ellof & Howen, 1980). Furthermore, these protists are typically larger in size due to their high predatory capacity (DeLong & Vasseur, 2012), making them relatively easier to observe in samples - a pattern that was not reflected in observations by certain analyzers.

Additionally, methodological issues, such as the use of fixatives, can alter the shape and structure of fragile-celled organisms, complicating their identification as their original morphology is lost (Kotyk et al., 2023). Consistent with the findings of Weisse & Montagnes (2022), our study highlights the need to increase the number of specialists with continued training in the identification and counting of freshwater protists. This would ensure greater accuracy in understanding their real diversity and density, and consequently, their role in aquatic ecosystem functioning. Furthermore, other solutions can be proposed, such as integrating morphological approaches with molecular techniques to complement analyses and ensure a more accurate assessment of biodiversity (Abraham et al., 2024), especially when organism morphology is compromised.

In addition, other ways of analyzing samples must be considered, such as *in vivo* analyses, which make it possible to better estimate the abundance and richness of species (Foissner, 1991). This counting method is important for better observation of organism structures, as it preserves the original size and shape of the organisms (Pfister et al. 1999). Moreover, in *in vivo* counts, all analyzers, with greater or lesser experience, can participate simultaneously in the analysis of each sample, sharing identifications and doubts, minimizing analyzer error. These errors linked to the less experienced analyzers are, in this way, compensated for by the analysis of those more experienced in the sample. With fixed samples, each analyzer looks at a different sample, contributing to differences in the recording of species and their abundance estimates and, therefore, compromising the project results (Lindström et al., 2002).

Conclusion

The results showed that the experience of the analyzers significantly influenced the accuracy of the analysis of the ciliated protist community. The more experienced analyzers recorded higher species richness and density compared to the less experienced, with losses in richness and density. This difference seems to be related to the difficulty in observing and identifying smaller ciliate groups. Considering that *in vivo* analyses allow the participation of several observers (with different levels of experience) in the analysis of each sample, minimizing errors associated with the observer, our results also suggest that *in vivo* analyses should be prioritized, aiming for a better estimate of the diversity and abundance of planktonic ciliates.

These findings highlight the relevance of continued training to minimize analytical biases and point to the need to integrate morphological and molecular approaches to improve the accuracy and reliability of the data. Such measures are essential to expand knowledge about the diversity of planktonic ciliated protists.

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