Acta Limnologica Brasiliensia

Publication of the Brazilian Association of Limnology

Thematic Section "Neotropical Zooplankton Symposium"
Acta Limnologica Brasiliensia, 2024, vol. 36, e103
https://doi.org/10.1590/S2179-975X6224
ISSN 2179-975X on-line version

Epistylid ciliates as epibionts on calanoid copepods in an Amazonian floodplain lake (Batata Lake)

Ciliados epistilídeos epibiontes em copépodes calanóides em um lago amazônico de planície de inundação (Lago Batata)

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Cite as: Flores, W.A. et al. Epistylid ciliates as epibionts on calanoid copepods in an Amazonian floodplain lake (Batata Lake). *Acta Limnologica Brasiliensia*, 2024, vol. 36, e103. https://doi.org/10.1590/S2179-975X6224

Abstract: Aim: The main objective was to investigate changes in the prevalence and infestation intensity of the epibiotic relationship (ciliates/calanoids) in relation to the impact of bauxite tailings (natural and impacted areas) during two distinct periods of the hydrological pulse (rising and high-water). **Methods:** The hydrological pulse of this system can be characterized with four distinct phases: rising, high-water, falling, and low-water. After degradation by bauxite tailings for ten years (1979-1989), and despite an ongoing long-term ecological restoration process, it is still possible to recognize two distinct regions in the lake: the area impacted by the tailings and the natural area. We sampled zooplankton (calanoids and epibiont ciliates) and limnological variables during the high-water period (March 2015) and rising-water period (June 2019) at twelve sampling points in Lake Batata, six in the impacted area and six in the non-impacted area. **Results:** This is the first record of peritrichous epibiont ciliates *Epistylis* sp. colonizing calanoid copepods in Amazonian ecosystems (Lake Batata, Pará, Brazil). We recorded epibiont ciliates in the calanoid copepodite and adult stages, as well as the main location sites on the thorax and abdomen. Our study recorded a significant difference in the prevalence and mean infestation intensity between the natural and bauxite tailings-impacted areas, and in the prevalence of infestation between the rising -water (2015) and high-water (2019) collections. The prevalence of infestation was higher in the impacted area and during the rising-water period. **Conclusions:** Although the host/epibiont/environment interaction is complex and requires analysis with a larger number of temporal samples, this study records a clear effect of flooding in this Amazonian system on the spatial and temporal dynamics of epibiont ciliates associated with calanoid copepods.

Keywords: bauxite tailings; epibiosis; *Epistylis*; flood pulse; Peritrichia.

Resumo: Objetivo: O objetivo principal foi investigar mudanças na prevalência e intensidade de infestação da relação epibiótica entre ciliados e calanóides em relação ao impacto por rejeito de bauxita (área natural e impactada) e em dois distintos períodos do pulso hidrológico (rising and high-water). **Métodos:** O pulso hidrológico desse sistema pode ser caracterizado por quatro fases distintas: enchente, águas-altas, vazante e águas-baixas. Após longa deposição de rejeitos de bauxita

(1979-1989), e apesar de um processo de restauração ecológica de longo prazo em andamento, ainda é possível reconhecer duas regiões distintas no lago: área impactada pelo rejeito e área natural. Nós amostramos o zooplâncton (calanóides e ciliados epibiontes) e as variáveis limnológicas durante os períodos de enchente (março de 2015) e de águas altas (junho de 2019) em doze pontos de coleta no Lago Batata, seis deles na área impactada e seis na área não impactada. **Resultados:** Este é o primeiro registro de ciliados epibiontes peritríqueos *Epistylis* sp. colonizando copépodes calanóides em ecossistemas amazônicos (Lago Batata, Pará, Brasil). Registramos os ciliados epibiontes nas fases de copepodito e adulto, bem como sítio de localização principal no tórax e no abdômen. Nosso estudo registrou significativa diferença entre a prevalência e intensidade média de infestação entre as áreas natural e impactada por rejeito de bauxita, e na prevalência de infestação entre as coletas realizadas na enchente (2015) e águas altas (2019). A prevalência de infestação foi maior na área impactada e no período de enchente. **Conclusões:** Embora interação hospedeiro/epibionte/ambiente seja complexa e necessite de uma análise com número maior de amostras temporais, esse estudo registra claro efeito da inundação nesse sistema amazônico sobre a dinâmica espacial e temporal dos ciliados epibiontes associados aos copépodes calanóides.

Palavras-chave: rejeito de bauxita; epibiose; *Epistylis*; pulso de inundação; Peritrichia.

1. Introduction

Epibionts are organisms that, during the sessile phase of their life cycle, colonize the surface of a living substrate. The basibiont serves as a substrate that hosts the epibiont (Wahl, 1989). According to Cook et al. (1998), epibiosis has two extremes: on one side, it is opportunistic, facultative, and non-specialized, while on the other, it involves obligatory and highly specific associations. Recent ecological studies on the subject support Wahl's (2008) proposal that epibiosis modulates interactions between the basibiont and the environment. Among metazoans, crustaceans are the organisms with the most recorded species of epibiont ciliates, particularly sessile peritrichous ciliates (Fernandez-Leborans & Tato-Porto, 2000; Fernandez-Leborans, 2009). Noteworthy studies on peritrichous ciliates on crustaceans investigate the following ecological aspects of the relationship between peritrichous ciliates and calanoid copepods: location site (Utz & Coats, 2005; Corre et al., 2020), specificity (Jones et al., 2016; Kumar et al., 2022), host density (Santos et al., 2020), spatial and temporal distribution (Utz & Coats, 2005; Cabral et al., 2017; Corre et al., 2020), environmental filters (Utz & Coats, 2005; Cabral et al., 2017; Corre et al., 2020), deleterious effects (Xu & Burns, 1991; Bickel et al., 2012; Souissi et al., 2013; Burris & Dam, 2014; Jones et al., 2016; Kumar et al., 2022), and the influence of the hydrological pulse on the dynamics of the ciliate-calanoid relationship (Cabral et al., 2017).

Lake Batata (1°28'S, 56°14'W) is a clear-water Amazonian lake connected to the right bank of the Trombetas River in Oriximiná, Pará, Brazil (see Cardoso et al., 2023), characterized by a marked flood cycle (depth varies between 2m and 8m). The lake has about 28 km^2 of permanent floodplain and is connected to the Trombetas River, a tributary of the Amazon River. The hydrological pulse of this system has four well-established phases: rising (March), high-water (June), falling (September), and low-water (December). This system received millions of tons of bauxite tailings between 1979 and 1988. After the deposition of bauxite tailings, two distinct regions can be recognized in the lake: the area impacted by the tailings and the natural area (Josué et al., 2021; Cardoso et al., 2023) (Figure 1). Over the past 36 years, Long-Term Ecological Research has been conducted in the lake to understand and implement a restoration process of this ecosystem. According to Cardoso et al. (2023), both the physicochemical variables of the water and the biological communities (viruses, bacteria, heterotrophic flagellates, phytoplankton, zooplankton, macroinvertebrates, and fish) studied in the area are affected by the impact of bauxite tailings and/or the hydrological pulse (rising, high-water, falling, and low-water).

The main objective of this work was to record epibiont ciliates on calanoid copepods in the lake Batata and to investigate changes in the prevalence and infestation intensity of the epibiotic relationship in relation to the presence of bauxite tailings and during two distinct periods of the hydrological pulse. Thus, we tested two hypotheses: (i) the prevalence and mean infestation intensity of the epibiotic relationship differ between the natural and bauxite tailings-impacted regions, being higher in the impacted area; and (ii) during the high-water period, there is a decrease in the prevalence and mean infestation intensity of the epibiotic relationship.

2. Material and Methods

In the present study, zooplankton (calanoids and ciliate epibionts) and limnological variables were

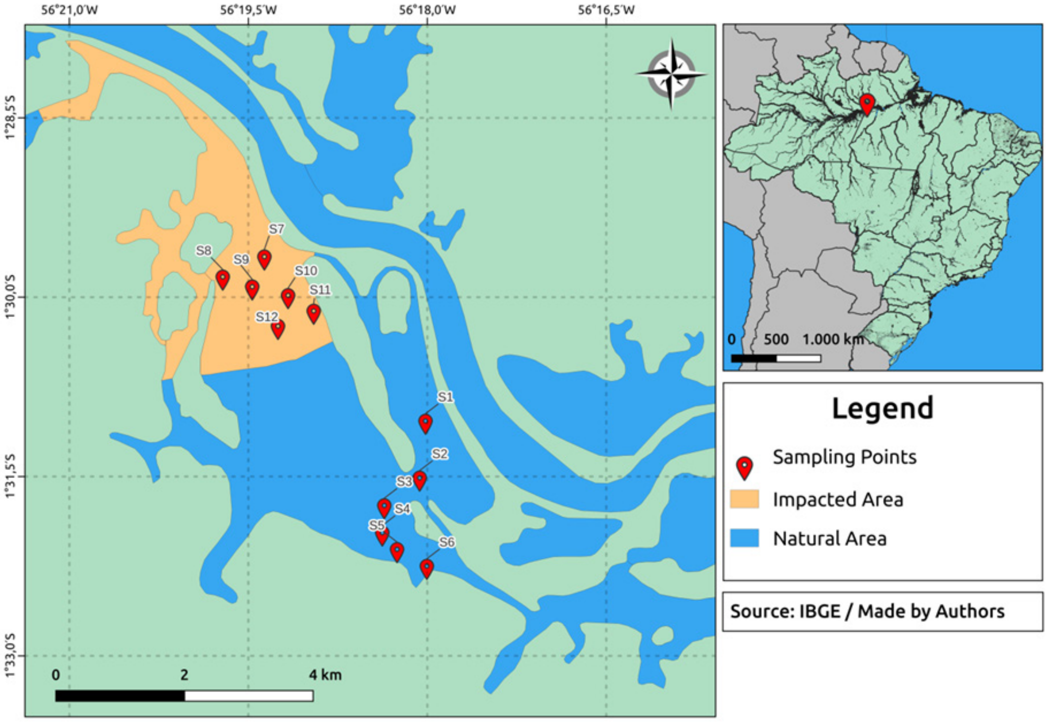

Figure 1. Map of the upper Batata Lake region (Oriximiná, northwest of the state of Pará, Brazil) showing the area impacted by bauxite tailings (S7-S12) and the natural area (S1-S6), as well as the sampling points in both regions.

sampled during the rising period (March 2015) and the high-water period (June 2019) at twelve sampling points in Lake Batata, six in the impacted area and six in the non-impacted area (Figure 1).

Water transparency was assessed using a Secchi disk. Water temperature, conductivity (μS/cm), and dissolved oxygen (DO) were measured in situ using a YSI 550A probe. In the laboratory, alkalinity (pH) was estimated by titration using H_2SO_4, and turbidity was measured using a LaMotte 2020 turbidimeter.

Zooplankton was sampled through vertical hauls (from near the bottom to the surface) using a plankton net (50 μm). The samples were preserved with 4% formalin solution (Haney & Hall, 1973), and the organisms were identified and counted in Sedgewick-Rafter chambers under a stereomicroscope. Copepods with epibionts were sorted and then photographed under an Olympus BX51 microscope with differential interference contrast at 40X and 60X magnification. The number of ciliate zooids on each copepod host was quantified. Copepods were identified as calanoids/cyclopoids, and immature (nauplii and copepodites) and mature copepods. In the 2015 campaign, mesozooplankton organisms were identified at the species level. The prevalence of infestation (number of hosts infested with ciliates/total number of hosts) and the intensity of infestation (number of ciliate zooids on each copepod) were recorded at the twelve sampling points during both sampling campaigns (2015 and 2019).

To identify patterns in the distribution of ciliate individuals, a series of statistical analyses was conducted. Initially, an exploratory analysis was performed. Boxplot graphs were constructed to examine the variation in the Average Epibiont Intensity (AEI) and prevalence between different areas and sampling dates. Then, a scatter plot was built to test the relationship between prevalence and AEI, as well as to observe the data distribution in relation to the years of collection and sampling areas. These analyses were carried out using the Python libraries Seaborn (Waskom, 2021) and Matplotlib (Hunter, 2007).

Next, one-way and two-way ANOVA tests were conducted to investigate whether there were significant variations in the values of prevalence and AEI between different areas and years of collection, both in isolation (one-way) and in combination (two-way). Both analyses were performed using the Python library Statsmodels (Seabold & Perktold, 2010).

Finally, a correlation analysis followed by a Redundancy Analysis (RDA) was conducted to investigate which physicochemical variables were related to the variation in prevalence and AEI and how these variables influenced the observed values. The correlation analysis was performed using the Python Matplotlib library, and the RDA was conducted in R using the Vegan package (Oksanen et al., 2020).

3. Results

During the March 2015 sampling, six species of copepods (calanoids, cyclopoids, and harpacticoids), 22 species of cladocerans, and 46 species of rotifers were recorded across twelve sampling points. Among these, ciliates were observed exclusively on the calanoid *Notodiaptomus coniferoides* Wright, 1927. Of the six copepod species identified, four were calanoids (*Aspinus acicularis* Brandorff, 1973, *Notodiaptomus coniferoides*, *Rhacodiaptomus besti* Santos-Silva & Robertson 1993, *Rhacodiaptomus retroflexus* Brandorff, 1973), one was a cyclopoid (*Oithona amazonica* Burckhardt, 1912), and one was an unidentified harpacticoid. In the June 2019 collection, ciliates were again restricted to calanoids, although not all species of copepods, cladocerans, and rotifers were identified. Consequently, ecological data were derived from the epibiotic relationship between ciliates and calanoid copepods during both collection periods across the twelve sampling points (Figure 2).

The sessile ciliates found colonizing the calanoid copepods in both of the sampling periods were identified as belonging to the genus *Epistylis* (Peritrichia, Epistylididae). This classification is due to the form of the zooid found which resemble the base form for the family Epistylididae and the lack of myonemes on the stalk. The zooids measure approximately 65 x 35 μm when fixed in formaldehyde (Figure 2). Despite attempts to amplify the 18S-rDNA using both universal (Medlin et al., 1988) and specific primers for Peritrichia (Souza et al., 2024), no amplification was successful.

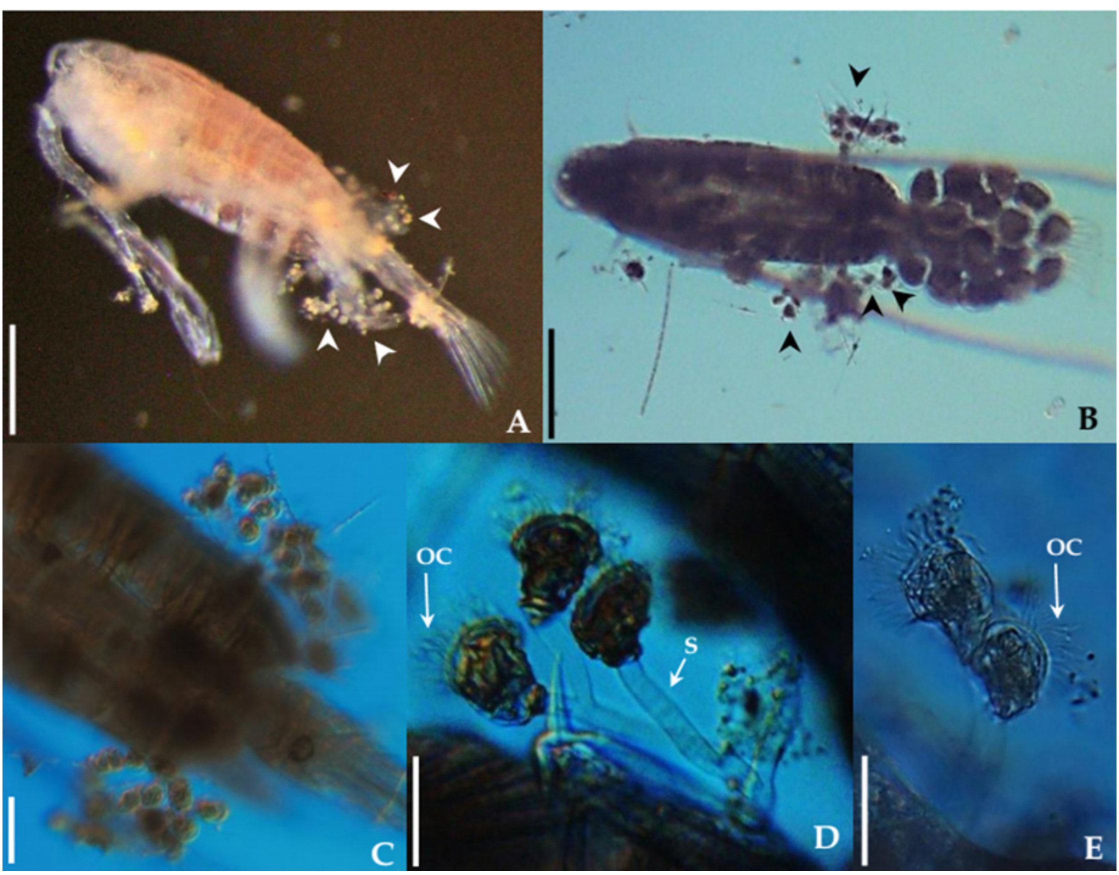

Figure 2: Photomicrographs of *Epistylis* sp. (Ciliophora, Peritrichia) colonizing copepods (Copepoda, Calanoida) from Batata Lake (Pará, Brazil), using differential interferential contrast (DIC). **A-C**. Colonization site in the dorsal region on thorax and abdomen segments (arrows indicate the ciliate location). **D-E**. Details of the morphology of the zooids and the peduncle (without myonemes). OC: oral ciliature; S: stalk. Bars: A-B = 350 μm; C = 150 μm; D-E = 75 μm.

No ciliates were found in association with the nauplius stage; they were present only on copepodite and adult stages. The primary attachment sites of the peritrichid ciliates on calanoids were the last thoracic (metasome) and proximal abdominal (urosome) segments (Figure 2).

Epistylis sp. was recorded in 17 out of 24 samples, with 12 (S1-S12) from 2015 and five (S7-S11) from 2019. Notably, no epibiotic ciliates were detected in copepods from the natural area in 2019. During the 2015 rising-water period, prevalence was 29.7% in the natural area and 44.9% in the impacted area, with a mean infestation intensity (AEI) of 18.8±8.75 in the natural area and 26.0±11.56 in the impacted area. In contrast, the 2019 high-water period showed a sharp decrease in prevalence, with 0% in the natural area and 8.11% in the impacted area, while AEI increased significantly to 46.65±16.67 in the impacted area (Figure 3B-C).

An exploratory scatter plot analysis (Figure 3A) revealed a relationship between epibiosis prevalence and intensity across natural and impacted areas in both 2015 and 2019. The data indicated higher prevalence and intensity in impacted areas during both years, with a marked increase in epibiosis intensity in 2019. One-way ANOVA indicated no significant difference in prevalence between areas (F: 1.78; p: 0.196), but a significant difference between years (F: 34.64; $p < 0.001$). For AEI, there was a significant difference between areas (F: 13.35; p: 0.001), but no difference between years (F: 0.00053; p: 0.982). Two-way ANOVA confirmed that AEI was significantly affected by area (F: 12.74; p: 0.002), but not by year (F: 0.0008; p: 0.977). For prevalence, both area (F: 5.014; p: 0.036) and year (F: 40.9667; $p < 0.001$) were significant factors.

To explore the influence of abiotic factors on epibiosis prevalence and intensity in Lake Batata, we analyzed physical-chemical data from both collection periods. A negative correlation was observed between prevalence and depth, while positive correlations were found with turbidity and conductivity (Figure 3F). For AEI, positive correlations were noted with temperature and pH (Figure 3E). Redundancy analysis (RDA) further clarified these relationships, explaining approximately 76% of the variation in prevalence and AEI through the analyzed physical-chemical parameters (Figure 3D). Higher conductivity and turbidity were associated with an increased likelihood of epibiosis, while greater Secchi depth correlated with higher prevalence. For AEI, increased pH and temperature were linked to a higher number of epibionts, whereas greater depth reduced both the likelihood of finding copepods with basibionts and the number of basibionts present (Figure 3D).

4. Discussion

This study is the first record of this epibiotic relationship between *Epistylis* sp. and copepods in Amazonian ecosystems (Lake Batata, Pará, Brazil). During the collection in March 2015, all zooplankton organisms were identified, revealing six species of copepods (calanoids, cyclopoids, and harpacticoids), 22 species of cladocerans, and 46 species of rotifers across twelve sampling points. Of the species present in the zooplankton, *Epistylis* sp. was recorded exclusively on the calanoid *Notodiaptomus coniferoides*. Among the six copepod species identified in 2015, four were calanoids (*Aspinus acicularis*, *Notodiaptomus coniferoides*, *Rhacodiaptomus besti*, *Rhacodiaptomus retroflexus*), one was a cyclopoid (*Oithona amazonica*), and one was a harpacticoid (unidentified). In the subsequent collection in June 2019, ciliates were again found only on calanoids, though not all copepods, cladocerans, and rotifers were identified at the species level. Consequently, ecological data were based on the epibiotic relationship between ciliates and calanoid copepods (Figure 2) for both collection periods at the twelve sampling points. Previous studies have suggested that peritrich ciliates exhibit species-specific preferences for copepod hosts, pointing to high specificity in these relationships (Utz & Coats, 2005; Jones et al., 2016; Cabral et al., 2017; Kumar et al., 2022). While this aspect was not the focus of our study, the data from 2015 suggest a similarly high specificity, thus requiring further investigation during other periods of the hydrological pulse in Lake Batata.

The recorded peritrich ciliates possessed a stalk without myonemes and an epistyliform zooid, measuring approximately 65 x 35 µm (formalin-fixed). Based on these morphological traits, they were identified as *Epistylis* sp. (Peritrichia, Epistylididae) (Figure 2). The fixation in formaldehyde prevented the observation of additional morphological details, and attempts to amplify the 18S-rDNA gene using both universal (Medlin et al., 1988) and specific primers for Peritrichia (Souza et al., 2024) were unsuccessful, making species-level identification impossible. The possibility that this is a new taxon cannot be dismissed, as it shares characteristics with epistylids found on copepods in the Paraná River floodplain (Cabral et al., 2017) and lakes in the Doce River basin (Santos et al., 2020) in Brazil.

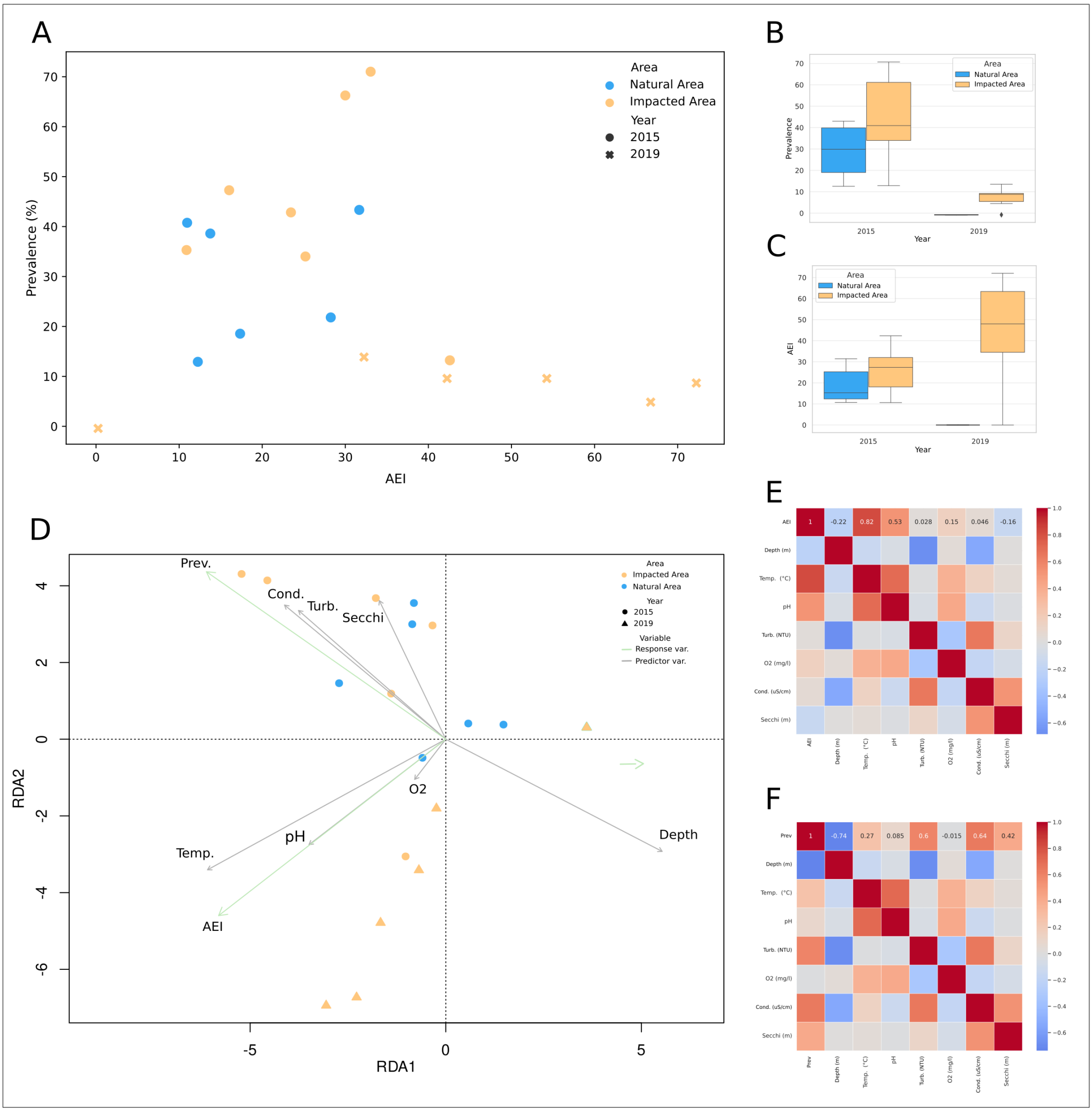

Figure 3. Prevalence and average epibiont intensity analysis (AEI) of *Epistylis* sp. (Ciliophora, Peritrichia) colonizing copepods (Copepoda, Calanoida) from Batata Lake (Pará, Brazil). **A.** Scatter plot showing the relationship between prevalence (%) and average epibiont intensity with information about area and sampling date; **B.** Boxplot showing the variance in prevalence (%) between areas and sampling dates; **C.** Boxplot showing the variance in average epibiont intensity between areas and sampling dates; **D.** RDA showing the relationship of the variance in prevalence and average epibiont intensity with the physical-chemical parameters; **E.** Heatmap showing the relationship of the average epibiont intensity and physical-chemical parameters. **F.** Heatmap showing the relationship of the prevalence and physical-chemical parameters. Abbreviations: AEI.: Average Epibiont Intensity; Cond: Conductivity (µS/cm); O_2: Dissolved Oxygen (mg/l); pH: "potential of hydrogen"; Prev: Prevalence (%); Secchi: Secchi depth (m); Temp.: Temperature (°C); Turb.: Turbidity (NTU).

Notably, no ciliates were associated with the nauplius stage of copepods; they were only observed on copepodite and adult stages, a pattern consistent with previous reports (Utz & Coats, 2005; Jones et al., 2016). Early developmental stages undergo more frequent molting, potentially reducing colonization opportunities, while more advanced stages offer more stable surfaces. Utz & Coats (2005) observed higher ciliate infestation in copepodites than in adults, whereas Jones et al. (2016) recorded a higher prevalence in adults. The factors, both intrinsic and extrinsic, that influence ciliate colonization on different developmental stages remain unclear and require further study.

The main attachment sites of *Epistylis* sp. on calanoid copepods were the last thoracic segments (metasome) and the abdominal segments (urosome) closest to the metasome (Figure 2). Other studies have found epibiotic peritrichs on the cephalothorax (prosome) and abdomen (urosome) of copepodites and adult copepods (Regali-Seleghim & Godinho, 2004; Corre et al., 2020). Utz & Coats (2005) recorded lower densities of peritrich ciliate zooids colonizing the legs and antennae of calanoid copepods compared to colonization on the cephalothorax and abdomen. Although Henebry & Ridgeway (1979) argued that ciliate attachment is random due to their bacterivorous nature, Green (1974) suggested that friction from appendages (legs and antennae) might explain the lower ciliate density in these areas on crustaceans, an idea supported by more recent studies (Regali-Seleghim & Godinho, 2004; Utz & Coats, 2005; Corre et al., 2020).

Our study demonstrates differences in prevalence and AEI between the natural and impacted stations, suggesting that higher infestation prevalence may be related to increased turbidity and electrical conductivity in the bauxite-impacted area. According to Cardoso et al. (2023), numerous limnological studies conducted in Lake Batata between 1988 and 2022 have shown that bauxite impact is associated with the deposition of inorganic particles, increased turbidity, decreased water transparency, altered nutrient cycling, and consequently, modifications in the dynamics, structure, and composition of the analyzed aquatic community: viruses, bacteria, phytoplankton, heterotrophic flagellates, zooplankton, macroinvertebrates, and fish. Due to the higher concentration of suspended clay in the water column in the bauxite-impacted area, Anesio et al. (1997) demonstrated that there is a higher density of bacteria adhered to suspended particles in this area. The mentioned authors did not observe a clear difference in the density of free-floating bacteria between the natural and impacted areas; however, they evidenced a difference in the bacterial community adhered to suspended particles, which are highly numerous in the impacted area. Since peritrich ciliates are predominantly bacterivores, the higher prevalence found in this study may be related to the primary food source of epibionts. The correlation of infestation prevalence with turbidity and electrical conductivity in our study corroborates a potentially higher density of adhered bacteria in the impacted area as reported by Anesio et al. (1997) in Lake Batata. Studies involving epibiotic peritrichs and invertebrate hosts have reported a positive correlation between bacterial density and the prevalence and/or intensity of infestation by epibiotic ciliates (Dias et al., 2009; Regali-Seleghim & Godinho, 2004; Cabral et al., 2010; Cabral et al., 2017; Cabral et al., 2018). Although correlations between variables do not necessarily imply a direct cause-and-effect relationship, future studies in Lake Batata on the epibiotic relationship could analyze the density of adhered and free-living bacteria in natural and impacted areas. In a study investigating the effect of turbidity on the density of epistylid epibionts on copepods of the species *Pseudodiaptomus stuhlmanni* Poppe & Mrázek, 1895, it was shown that turbidity can play an important role in modulating this epibiotic interaction, as higher prevalence and density of epibionts in turbid environments may negatively impact host longevity (Jones et al., 2018). Our results about spatial distribution (natural and impacted area) suggest that epibiotic relationships between ciliates and calanoids were more frequent in the impacted area, a pattern previously reported in studies showing that epibiotic relationships tend to be more common and severe in altered habitats (Henebry & Ridgeway, 1979; Xu, 1992; Cabral et al., 2017).

In addition to the spatial difference (natural and impacted areas) recorded for epistylid ciliates on calanoid copepods in Lake Batata, we observed a decrease in infestation prevalence during high-water periods, although no difference in epibiont abundance was found between the two periods analyzed. Previous studies have demonstrated the effect of temporal dynamics on the prevalence and infestation intensity of peritrich epibionts on copepods (Regali-Seleghim & Godinho, 2004; Utz & Coats, 2005; Santos et al., 2020; Corre et al., 2020), but studies in floodplains (Cabral et al., 2017) where the hydrological pulse is highly heterogeneous are rare. In the floodplain of the Paraná River, during the rainy season (potamophase), there were changes in the abiotic conditions of the system (turbidity, nutrients, chlorophyll), leading to changes in bacterial density (food) and copepods, resulting in reduced infestation prevalence (Cabral et al., 2017). In Lake Batata, during high-water periods, there is a large influx of water from the Trombetas River, resulting in greater homogeneity in the physicochemical parameters of the water compared to low-water periods (Thomaz et al., 2007). According to Anesio et al. (1997), there is a significant reduction in bacterial abundance during high-water periods in Lake Batata, likely due to dilution and reduced input of available dissolved organic carbon from the floodplain. Our study supports a decrease in epibiont infestation prevalence during high-water periods; however, we emphasize the need for a more robust temporal study in the region, analyzing all hydrological pulses (rising, high-water, falling, and low-water) over one or more consecutive cycles. Several studies in Lake Batata have documented the effects of flooding on limnological parameters and changes (abundance and/or composition) in communities of viruses, bacteria, phytoplankton, zooplankton, macroinvertebrates, fish, aquatic plants, and functional diversity (Cardoso et al., 2023), yet there have been no studies on this effect on epibiotic communities.

Our study is the first to document epistylid ciliates associated with zooplankton components (calanoid copepodites and adults) in Lake Batata, Amazon. We found epibiotic ciliates primarily on the thorax and abdomen of copepods, with no presence on their legs and antennae. In line with our initial hypotheses, we observed significant differences in prevalence and mean infestation intensity between natural and bauxite-impacted areas. Additionally, we confirmed a decrease in the prevalence of epibiotic infestation during high-water periods. However, contrary to our expectations, the abundance of epibionts did not differ significantly between the two periods analyzed. These results suggest that while the host/epibiont/environment interaction is influenced by environmental factors such as water level, the abundance of epibionts may be governed by more complex dynamics. Thus, broader temporal sampling is required to better understand the effect of flooding on epibiotic ciliates and their impact on calanoid hosts.

Acknowledgements

We thank Mineração Rio Norte S.A. for fieldwork support. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; fellowships to WAF and PMS) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, project 314070/2021-4 and fellowships to RJPD and RLB).

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Received: 29 June 2024
Accepted: 14 October 2024

Associate Editors: Claudia Bonecker, Gilmar Perbiche-Neves, Maria Stela Maioli Castilho Noll.