FEDERAL UNIVERSITY OF ITAJUBÁ

NATURAL RESOURCES INSTITUTE

POSTGRADUATE PROGRAM IN ENVIRONMENT AND WATER RESOURCES

PROTIST DIVERSITY OF THE ATLANTIC FOREST IN SOUTHEASTERN BRAZIL VIA DNA METABARCODING

VANESSA CARVALHO DA SILVA

Itajubá (MG), Brazil

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Dissertation submitted to the Postgraduate Program in Environment and Water Resources, as part of the requirements for obtaining the title of Master in Environment and Water Resources.

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To my family, especially to my mother, for all the support provided. And to my children as my main motivators.

ABSTRACT

Protists are highly diverse unicellular microeukaryotes found in a wide range of habitats. They play important roles in nutrient cycling and ecosystem maintenance. However, much of protist diversity remains unknown, particularly in the Neotropical region. Due to their high sensitivity to environmental changes, these organisms are widely used as biological indicators of organic pollution. Therefore, understanding the taxonomic and functional diversity of these organisms is urgently needed, not only to fill gaps in our knowledge but also to enable the development of public policies for biological conservation. In this study, using high-throughput sequencing of the gene encoding the V4 region of the 18S rRNA gene through DNA metabarcoding technique, we investigated the taxonomic and trophic diversity of the main groups of protists in freshwater systems and brackish coastal lagoons located in fragments of the Brazilian Atlantic Forest. The protist communities of coastal lagoons were as diverse as the freshwater systems studied in terms of alpha diversity, differing significantly in terms of taxonomic composition. Remarkable functional homogeneity was observed among trophic groups in freshwater environments. Beta diversity was higher among freshwater samples, suggesting a higher level of heterogeneity regarding the composition and abundance of OTUs. Ciliophora was the most represented group in freshwater, while Diatoms dominated diversity in coastal lagoons. Subsequently, using the same technique, the molecular diversity of ciliated protists was investigated at seven strategic points along the Sapucaí River (Itajubá, Minas Gerais, Brazil) to assess the impact of urban pollution on the richness, abundance, and diversity indices of these communities. For each sampling point, values of physico-chemical parameters were also recorded. The composition of ciliates varied significantly along the course of the Sapucaí River. Samples collected in urban areas showed lower richness and diversity, corroborating the influence of pollution gradient on these communities. Physico-chemical parameters showed little variation between samples and were not linked to observed changes in ciliate communities, revealing that these organisms are strongly affected by environmental changes and respond more sensitively to these disturbances than physico-chemical parameters, emphasizing their potential as bioindicators.

Keywords: DNA metabarcoding, Neotropics, Protist diversity, Ciliophora, Biomonitoring.

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LIST OF ABBREVIATIONS AND ACRONYMS

°C - Degrees Celsius

bp - Base pairs

DNA - Deoxyribonucleic Acid

EC - Electrical conductivity

ENA - European Nucleotide Archive

HTS - High-Throughput Sequencing

NMDS - Non-metric Multidimensional Scaling

ORP - Oxidation-reduction potential

OTUs - Operational Taxonomic Units

PCoA - Principal Coordinate Analysis

PCR - Polymerase Chain Reaction

PES - Polyethersulfone

pH - Potential of hydrogen

QIIME - Quantitative Insights Into Microbial Ecology

rRNA - Ribosomal Ribonucleic Acid

TDS - Total dissolved solids

SUMMARY

GENERAL INTRODUCTION

Much of Earth's diversity is dominated by protists, a paraphyletic group of cosmopolitan unicellular eukaryotic organisms with varied morphology and characteristics distributed among numerous phyla, with medical, biotechnological, environmental, and other importance (Tortora; Case; Funke, 2012). In addition to soil, free-living protists are abundant in rivers, lakes, and oceans, including planktonic, benthic, and physically and chemically adverse conditions such as temperature and salinity (Corliss, 2001). There are also symbiotic species that inhabit various hosts through mutualism, commensalism, parasitism, or phoresy (Corliss, 2002). They are essential elements of food webs in all environments where they are found, acting as predators of bacteria and other protists and serving as prey for metazoans, as well as being involved in biogeochemical cycles (Fenchel, 1987). As an example, autotrophic algae in aquatic environments have carbon fixation capabilities comparable to terrestrial plants (Worden et al., 2015). In addition to heterotrophs and autotrophs, there are mixotrophic organisms that can perform phagotrophy and phototropism (Jones, 2000).

Despite the important role that protists play in the dynamics of ecological processes, there is a gap in the knowledge of their diversity and abundance (del Campo et al., 2014). Studying the composition and structure of protist communities provides important information about the impacts of pollution and other human activities on ecosystems (Baird & Hajibabaei, 2012). Different taxa of organisms are used as bioindicators, with a focus on macroinvertebrates. However, the main advantages of protists over other organisms as indicators are their high environmental sensitivity, functional importance, wide distribution and abundance, small size, and short generation time (Payne, 2013). Four protist taxa are most used for biomonitoring studies as ecological indicators: ciliates, diatoms, foraminifera, and testate amoebae (Pawlowski et al., 2016).

Ciliate protists are heterotrophic organisms characterized by nuclear dimorphism, sexual reproduction by conjugation, and complex infraciliature (Adl et al., 2019). There are many parasitic, commensal, mutualistic, and mixotrophic species (Esteban et al., 2010). They have a wide geographical distribution, occupying aquatic and terrestrial environments, as well as mosses and bromeliad tanks (Singer et al., 2021; Foissner 1996; Simão et al., 2017). They are often used in studies assessing the quality of continental waters in Europe (Madoni & Bassanini, 1999; Madoni & Braghiroli, 2007; Quintela-Alonso et al., 2018) due to these organisms' ability to quickly reflect the effects of pollution on aquatic ecosystems (Américo-Pinheiro et al., 2017). However, they are still poorly explored in the neotropical region, despite

being one of the most diverse regions on the globe (Ritter et al., 2021). The Atlantic Forest, for example, is the second largest tropical rainforest in the Americas. Although it is intensely studied regarding forest density and terrestrial fauna, its microbiota is poorly known (Tabarelli et al., 2005).

This scenario has been changing with the advancements of molecular techniques such as high-throughput sequencing (HTS) and DNA metabarcoding, enabling advances in taxonomic identification, phylogenetic analyses, diversity analyses, and biogeographic patterns of microbial communities (Rodríguez-Ezpeleta et al., 2021). According to Santoferrara et al. (2020), more than 350 articles using metabarcoding approaches that include protists have been published so far. Despite limitations, the applications of this technique in studies of molecular diversity of protists have shown promise.

By using high-throughput sequencing of the gene encoding the V4 region of the 18S rRNA gene through the DNA metabarcoding technique, the present study firstly aimed to investigate the taxonomic and trophic diversity of protists in freshwater systems and brackish coastal lagoons in fragments of the Atlantic Forest in order to understand the dynamics of the ecosystems in which they occur and contribute to filling the gap in knowledge of these communities. Secondly, the molecular diversity of ciliate protists along the Sapucaí River (Itajubá, Minas Gerais State, Brazil), representing a eutrophication gradient, was investigated to assess the impact of urban pollution on the richness, abundance, and diversity of these communities.

CHAPTER 1

PROTIST TAXONOMIC AND FUNCTIONAL DIVERSITY IN AQUATIC ECOSYSTEMS OF THE BRAZILIAN ATLANTIC FOREST

1.1. INTRODUCTION

It is widely known that microorganisms dominate the diversity on Earth and the protists, a paraphyletic assemblage of single-celled organisms, represent a significant part of this diversity (Adl et al., 2019; Burki et al., 2020). Protists can be found in a variety of habitats, often representing the largest portion of eukaryotic richness (de Vargas et al., 2015; Mahé et al., 2017; Singer et al., 2019; Obiol et al., 2020). Although they are very common, present in virtually all environments, molecular surveys of biodiversity has revealed that most of the taxonomic diversity of protists remains undescribed (Bass & Boenigk, 2011; Pawlowski et al., 2012; del Campo et al., 2014). This is especially evident in less explored regions such as the Neotropics (Dunthorn et al., 2012; Lentendu et al., 2019; Fernandes et al., 2021; Ritter et al., 2021; Câmara et al., 2022). This gap in knowledge about the taxonomic and functional diversity of protists is an obstacle to a clearer view of how ecosystems operate (Sherr et al., 2007; López-García & Moreira, 2008).

Protists are key components in the ecosystems they inhabit. They can be found as free-living forms, but many species are symbionts (i.e., parasites, parasitoids, mutualists and commensals) on a wide range of hosts, including other protists, plants and metazoans, directly affecting the ecological aspects and controlling populations of their hosts (Chambouvet et al., 2008; Nowack & Melkonian, 2010; Edgcomb, 2016). These parasitic and mutualistic symbionts can dominate the diversity and abundance in several environments (Guillou et al., 2008; Geisen et al., 2015; Mahé et al., 2017). In addition to being a source of food for many organisms, protists act at the base of food chains as primary producers, as consumers of bacteria or other protists, and as decomposers (Wetzel, 2001; Corliss, 2002). There are also protists capable of performing both photosynthesis and phagotrophy, according to their life cycle stage or environmental conditions, in a type of nutrition called mixotrophy (Jones, 2001; Mitra et al., 2014). Despite the importance of understanding the functional profile of protist communities in different environments (Singer et al., 2021), this type of investigation has rarely been done in Brazilian ecosystems (de Araujo et al., 2018).

The constant variation of the physico-chemical conditions and an overlap with the microbial communities from the adjacent soil could favor the development of a highly diverse

and dynamic protistan assemblage in freshwater systems (Debroas et al., 2017; Boenigk et al., 2018). However, molecular studies have shown that transitional environments, such as brackish coastal lagoons and estuaries, also have high protist diversity (Schubert et al., 2011; Telesh et al., 2011; Telesh, Schubert & Skarlato, 2013; Grinienė et al., 2019), contrary to what was previously believed (Remane, 1934).

High-throughput sequencing of molecular markers from environmental samples, known as metabarcoding, are powerful tools to describe the diversity of protists (de Vargas et al., 2015) and have expanded our knowledge about the phylogenetic placement of these organisms and uncovered a high number of new lineages (Jamy et al., 2020; Rajter & Dunthorn, 2021; Czech et al., 2022). In under-explored regions with high potential for discovering new taxa, such as the Brazilian biomes (Fernandes et al., 2021; Câmara et al., 2022), this tool is even more promising.

The Atlantic Forest is one of the top two Brazilian ecosystems richest in plant and animal diversity and endemism (Mittermeier et al., 1999) and the world's fourth leading biodiversity hotspot (Myers et al., 2000). At the same time, this is one of the global most depleted habitats, retaining only a small part of its primary vegetation (Mittermeier et al., 1999). A number of associated habitats such as mangroves, rivers, streams, creeks, lakes, and lagoons are included in this biome. More than 90% of the Atlantic Forest is within the Brazilian territory, therefore, its conservation is largely a Brazilian concern (Marques & Grelle, 2021). While the diversity of plants and vertebrates is relatively well documented, little is known about its microbial diversity (Pontes, 2015; Ritter et al., 2021). To the best of our knowledge, only two article have been published so far on the molecular diversity of protists in the Brazilian Atlantic Forest through DNA metabarcoding and both dealt exclusively with the diversity of the phylum Ciliophora (Simão et al., 2017; Fernandes et al., 2021). This is the first study to examine the taxonomic and trophic diversity of the major protist groups in water bodies located in the Atlantic Forest by DNA metabarcoding. We compared the α and β diversity among samples for the overall protists communities and assessed the relative abundance of phototrophic, consumers, and parasitic taxa in brackish coastal lagoons and freshwater systems, also contributing to a better understanding of the dynamics and adaptations of protists to different salinity levels.

1.2. MATERIALS AND METHODS

1.2.1. Sampling

Samples of freshwater and brackish water were obtained from 23 sites located in fragments of the Atlantic Forest in Rio de Janeiro state, Brazil (Fig. 1.1), as detailed in Fernandes et al. (2021). Five aliquots of 200 mL of water and resuspended sediment were collected along the edges of each sampling site, making up a total volume of 1 L per sample. The samples were stored in sterile plastic containers and then taken to the laboratory for filtration and DNA extraction less than 24 h after sampling. The total volume was filtered with a peristaltic pump through 0.22 µm Polyethersulfone (PES) membranes (75 mm diameter) and the retained content (about 0.5 g) was immediately processed for DNA extraction, ensuring the integrity of the microbial community. Negative field controls (sterilized water collected using the same protocol and equipment) were also obtained and processed in the same way as field samples to monitor possible contamination.

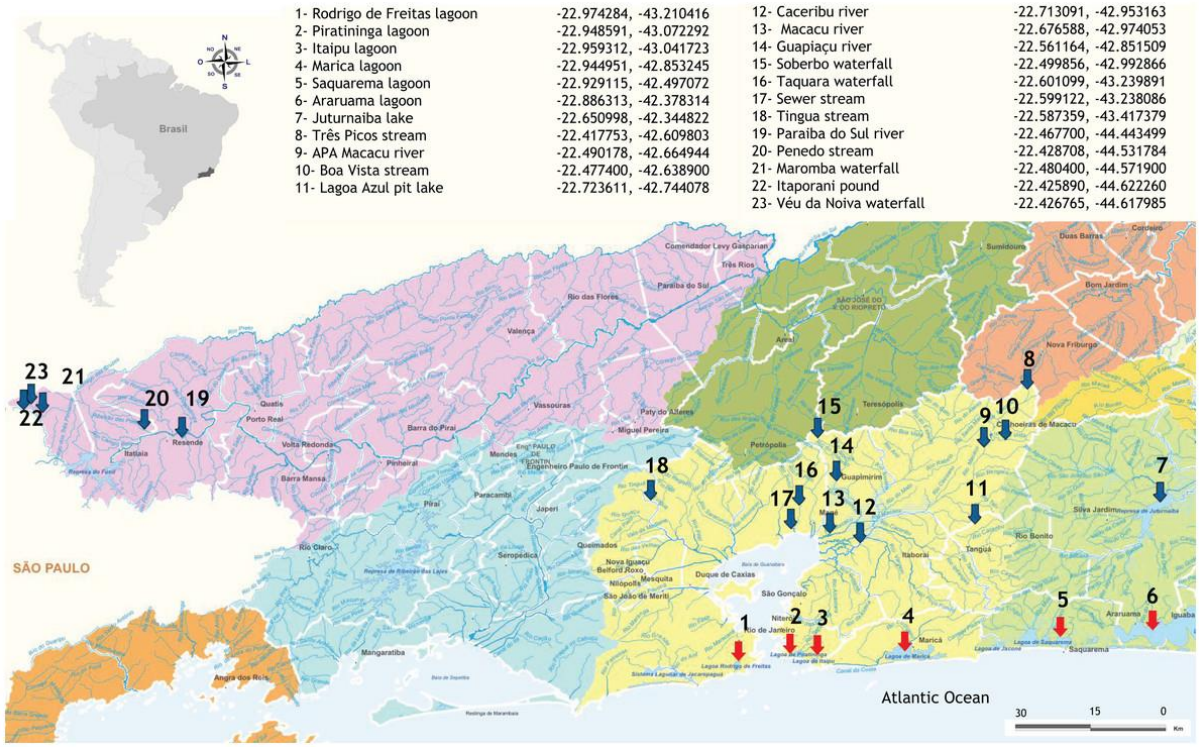

Figure 1.1 - Distribution and geographical coordinates of the aquatic ecosystems investigated in fragments of Atlantic Forest, Rio de Janeiro State, Brazil. Red arrows indicate brackish coastal lagoons and blue arrows freshwater environments.

1.2.2. DNA extraction and Illumina library construction

Total DNA extraction was performed using the PowerSoil® DNA Isolation kit (MoBio Laboratories, Carlsbad, CA USA). DNA yields were measured using the Qubit® 2.0 Fluorometer (Thermo Scientific, Waltha, MA, USA). The universal primers 528F (5′-GCG GTA ATT CCA GCT CCA A-3′) and 706R (5′-AAT CCR AGA ATT TCA CCT CT-3′) (Elwood, Olsen & Sogin, 1985; Cheung et al., 2010) were used to amplify the V4 region of the eukaryotic 18S rRNA gene in PCR reactions with the Phusion® High-Fidelity PCR Master Mix (New England Biolabs, USA). The amplicons were sequenced with an Illumina HiSeq 2500 sequencer (Illumina Inc., San Diego, CA, USA), and 2 × 250 bp reads were generated. Raw sequences are available through the project number PRJEB37554 on the European Nucleotide Archive (ENA).

1.2.3. Bioinformatics analyses

Sequencing reads from all samples were first merged with Flash v1.2.11 (Magoč & Salzberg, 2011) and then processed with Quantitative Insights Into Microbial Ecology 2—QIIME2 2022.2 (Bolyen et al., 2019) for demultiplexing and remotion of adaptors, using the q2-demux and q2-cutadapt (Martin, 2011) plugins. The reads were filtered to a minimum Phred quality score of Q20, denoised, dereplicated, and chimerical sequences were eliminated using the q2-quality-filter (Bokulich et al., 2013) and the q2-dada2 plugins (Callahan et al., 2016), respectively. Reads shorter than 210 bp length were also discarded. The amplicon sequence variants were clustered into operational taxonomic units (OTUs) using the q2-vsearch plugin (Rognes et al., 2016) and the open-reference method (Rideout et al., 2014) against the SILVA reference database version 138 (Quast et al., 2012). Sequences with ≥97% similarity were assigned to the same OTU. A sklearn classifier pre-trained on SILVA 138, region 515F/806R, was used to the taxonomic annotation of OTUs (Bokulich et al., 2018) with the q2-feature-classifier plugin (Pedregosa et al., 2011). OTUs from putative multicellular organisms (i.e., assigned to Metazoa, Embryophyta and Fungi) were removed, as well as the ones represented by less than 10 sequences, for noise reduction (Behnke et al., 2011).

1.2.4. Functional assignments of OTUs

The obtained taxonomy table was manually verified and OTUs were assigned to three major functional groups following Singer et al. (2021) as consumers (Ciliophora, Rhizaria, Obazoa non-Ichthyosporea, CRUMs, Amoebozoa, non-Ochrophyta, non-Peronosporomycetes

Stramenopiles and Centrohelida), phototrophic (Archaeplastida, Ochrophyta, Prymnesiophyceae and Cryptophyceae) and parasitic (Apicomplexa, Ichtyosporea, Peronosporomycetes, Phytomyxea, Perkinsidae, Syndiniales and Rozellomycota). Since these groups may include organisms with different functional roles, we analyze each OTU classified and consider the least inclusive taxonomic level to assign function. Some groups of Chrysophyceae have lost their photosynthetic ability secondarily (Dorrell et al., 2019). Therefore, we considered as consumers those OTUs assigned to *Oikomonas, Spumella, Apoikia, Poteriospumella* and *Paraphysomonas*, also following Singer et al. (2021). Other genera were considered phototrophic and the OTUs not classified at this level were tagged with unknown function.

1.2.5. Diversity studies

We estimated the α diversity, i.e., the number of observed OTUs, the Shannon's index H′ (Whittaker, 1972), and the Simpson's index D (Simpson, 1949) for each sample with the R-package phyloseq (McMurdie & Holmes, 2013). OTU richness in freshwater and brackish samples was also estimated using species accumulation curves (functions specaccum, R-package vegan v. 2.6–2) (Oksanen et al., 2022). We assessed the similarity patterns among protist communities (β diversity) using principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities obtained from the composition and relative abundance of sequences. Significance of differences between groups was assessed using the Permanova test (adonis function R-package vegan with 1,000 permutations). We tested for differences between ecosystems for α and β diversity indices by pairwise tests for multiple comparisons of mean rank sums (Nemenyi test, $p < 0.05$; function NemenyiTest, R package DescTools). We also use this approach to test the differences between functional groups based on the relative abundance of OTUs.

1.3. RESULTS

1.3.1. Protist community richness and heterogeneity in freshwater and brackish systems from Atlantic Forest

The sequencing generated a total of 1,742,075 reads. After all quality filtering steps, 253,637 reads with an average sequence length of 350 bp remained for downstream analysis. After clustering at 97% similarity, a total of 2,692 OTUs were retrieved. Subsequently, OTUs not assigned to the phylum taxonomic category, identified as 'unclassified', 'uncultured' and

‘incertae sedis’ (256 OTUs) were removed, as well as sequences from putative non-protist organisms, as OTUs assigned to Metazoa (383 OTUs), Fungi (408 OTUs) and Embryophyta (55 OTUs). In the end, a total of 1,590 OTU sequences assigned to protist groups were retained and used for the diversity analyses.

OTU richness tended to approach a saturation plateau, as shown by the species accumulation curves (Fig. 1.2A). Protist richness was significantly higher in freshwater (1,148 OTUs) than in brackish samples (419 OTUs). Only 23 OTUs were shared between these two sampling groups (Fig. 1.2B). However, when abundance data are considered, the means of the α diversity indices do not differ significantly between freshwater and brackish environments by t-test (Fig. 1.2C), suggesting that a more even distribution of sampling effort could equalize the OTU richness retrieved from these environments. The samples with the highest OTU richness were from Guapiaçu river (265 OTUs) and from Três Picos Park (272 OTUs). The sample with the highest α diversity value was from the Boa Vista stream. All these highly diverse sites are located in the Serra dos Órgãos National Park.

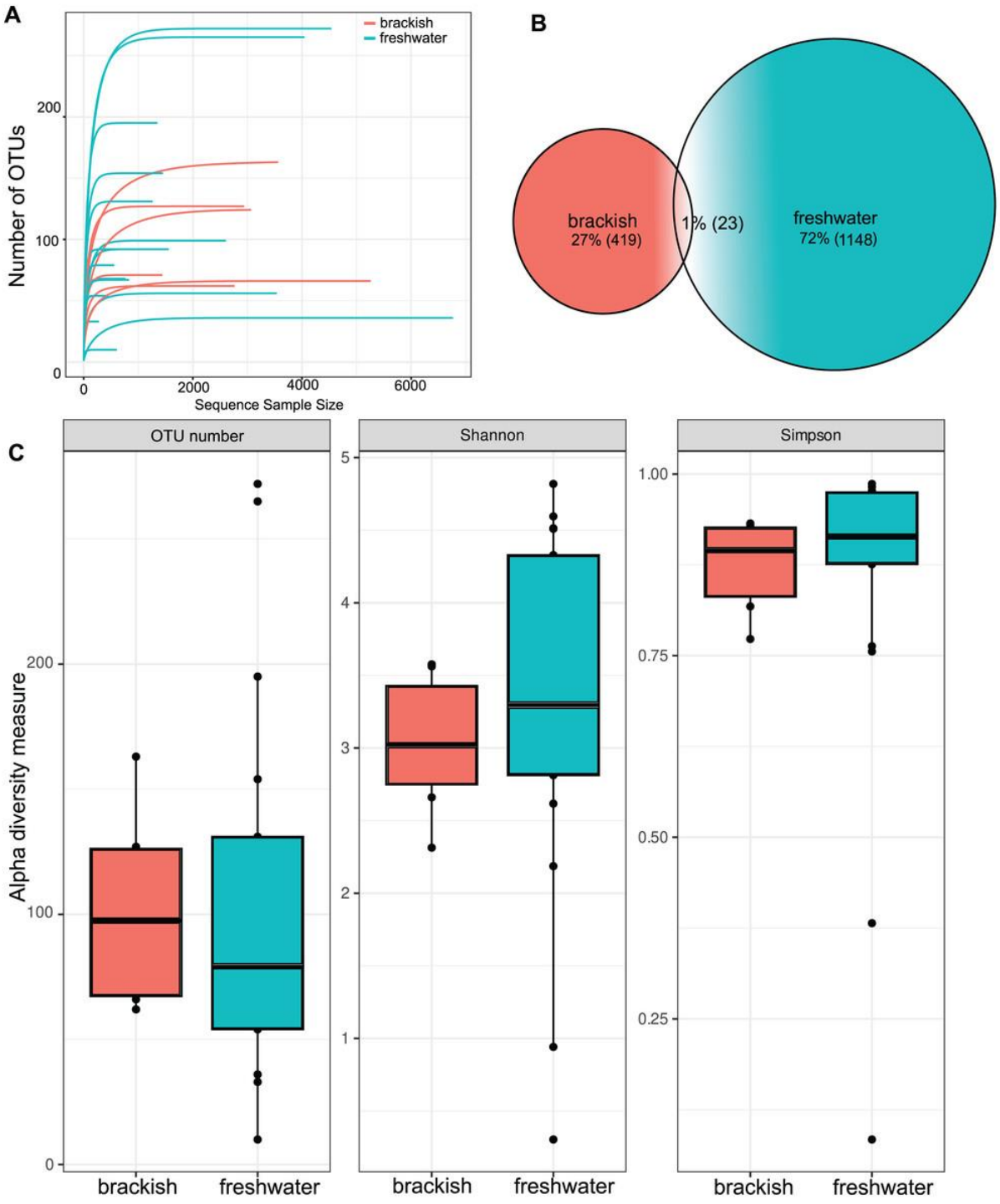

Figure 1.2 - Richness and diversity of protist OTUs. (A) Species accumulation curves by sample. (B) Venn's diagram of the total amount of OTUs in freshwater, brackish water and shared by both sample groups. (C) α diversity metrics. Number of unique OTUs; Shannon = Shannon's index H; Simpson = Simpson's index D. The average values of the α diversity indexes do not differ significantly between freshwater and brackish sampling groups (p-value > 0.05).

Beta diversity was highest among freshwater samples (0.968 ± 0.06) and significantly lower among brackish water samples (0.911 ± 0.15) (Fig. 1.3A). Principal Coordinate Analysis

revealed that protist communities from brackish and freshwater environments are distinctly structured (Fig. 1.3B). The adonis test showed that the richness and abundance of the protist OTUs are significantly different between freshwater and brackish samples (p-value < 0.01; Fig. 1.3B). This dissimilarity between the two environments with respect to the protist communities was also confirmed by the Nemenyi's test for multiple comparisons (p-value = 0.0014).

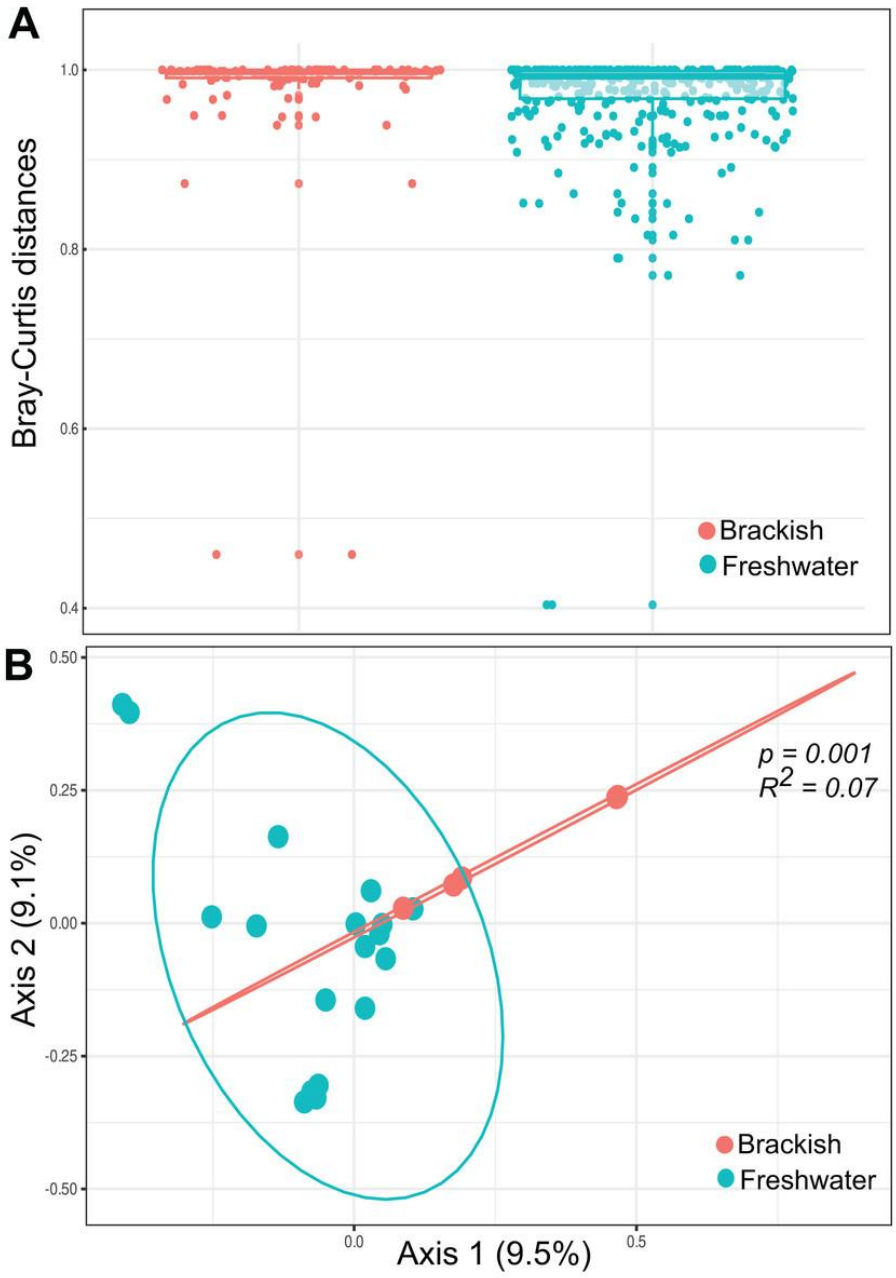

Figure 1.3 - Beta diversity measures. (A) Bray-Curtis distances within each ecosystem based on protist OTU composition (presence-absence data) and relative abundances. (B) Ordination plot (principal coordinates analysis = PCoA) of protists communities based on Bray-Curtis dissimilarities. The protist OTU composition in freshwater and brackish samples differs significantly (p-value < 0.01).

1.3.2. Taxonomic and functional diversity

The 1,590 OTUs were distributed among seven of the protist supergroups (sensu Burki et al., 2020). As expected, most of the sequences were assigned to the clade TSAR (1,292 OTUs), representing more than 80% of the total diversity. Archaeplastida (177 OTUs) followed with 11% of the total OTU diversity. The other groups were much less represented, such as Obazoa (44 OTUs), Amoebozoa (31 OTUs), Cryptista (23 OTUs), CruMs (13 OTUs), and Haptista (9 OTUs).

These OTUs were assigned to 26 major protist phyla (Fig. 1.4). Ciliophora is the most represented (451 OTUs), followed by Diatomea (336 OTUs), Chlorophyta (161 OTUs) and Cercozoa (153 OTUs), together accounting for over two-thirds of the sequences. The relative abundance in brackish lagoons is dominated by Diatomea, Ciliophora and Dinoflagellata. Other major protist lineages are relatively more abundant or exclusive to freshwater (Fig. 1.4). Diatomea was the only group with higher OTU richness in brackish water (Fig. 1.4). The most represented at the genus level were the bacillariophycean diatoms *Navicula*, *Amphora*, *Pinnularia* and *Nitzschia* with more than 20 OTUs each. A single OTU assigned to a marine haptophyte of the genus *Isochrysis* was detected exclusively in brackish samples. From the total, only 418 OTUs were detected in relative abundances ≥1%. This represents 26.3% of the total data set. Some of these OTUs showed relative abundances greater than 20% in the samples (Table 1.1). In particular, ciliates of the genera *Paramecium* and *Laurentiella* showed relative abundances of 96% and 80%, respectively, in some freshwater samples. Overall, ciliates are among the top five most abundant protists in the Brazilian Atlantic Forest (Fig. 1.4).

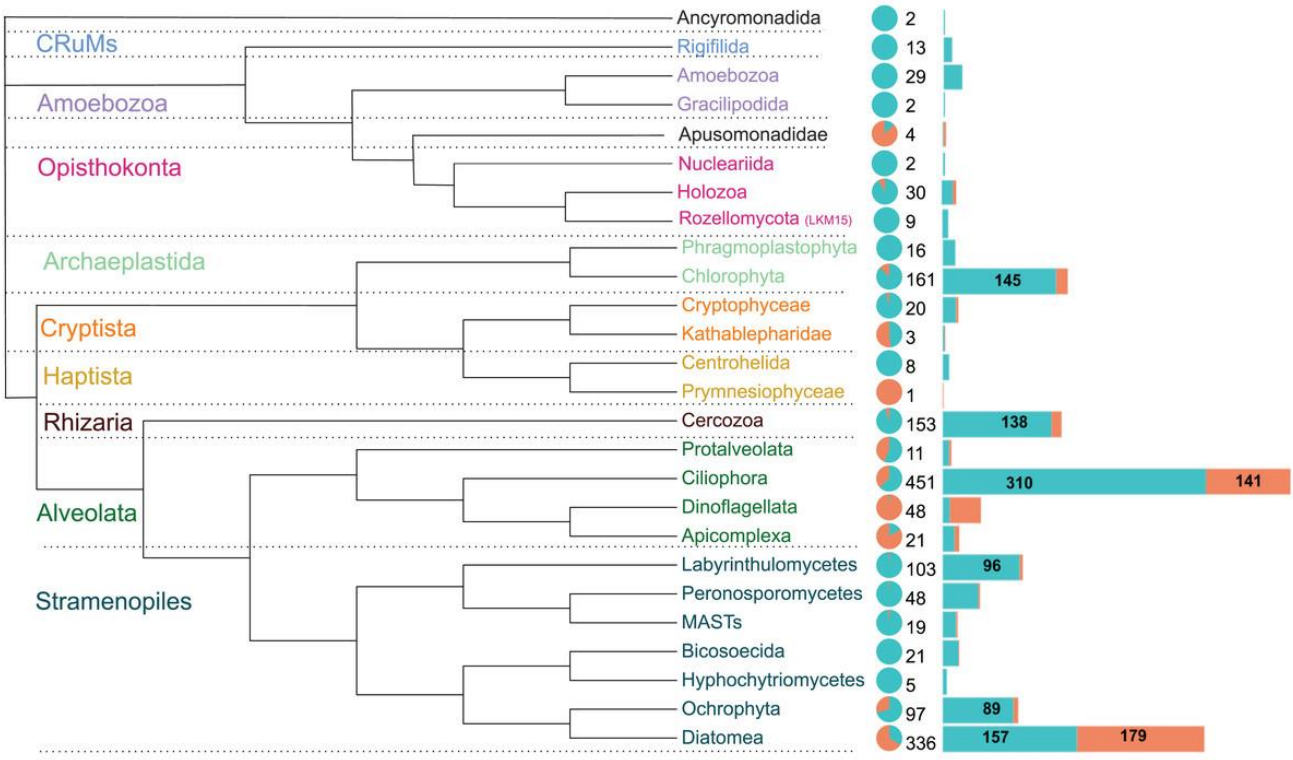

Figure 1.4 - Schematic phylogenetic tree of the main protist lineages, their relative abundances and OTU richness in freshwater (cyan) and coastal (coral) aquatic ecosystems of the Atlantic Forest. The pie chart represents the relative abundance of reads. Numbers at the right of the pie chart are the total OTU richness of each taxon and the barplots represent the distribution of these OTUs in each ecosystem. Protist groups with highest OTUs richness are indicated numerically. Ciliophora dominates the diversity in freshwater systems while Diatomea is the richest and most abundant group in brackish waters.

Table 1.1 - OTUs whose abundance exceeded 20% in the samples.

Protist main taxa	**Identification at genus rank**	**Environment of highest abundance**	**Highest abundance (%)**
Ciliophora	*Paramecium*	Freshwater	0.96
Ciliophora	*Laurentiella*	Freshwater	0.80
Ciliophora	*Frontonia*	Freshwater	0.65
Ciliophora	*Blepharisma*	Freshwater	0.53
Ciliophora	*Zoothamnium*	Brackish	0.48
Labyrinthulomycetes	*Labyrinthula*	Freshwater	0.46
Diatomea	*Synedra*	Freshwater	0.44
Dinoflagellata	*Blixaea*	Brackish	0.42
Phragmoplastophyta	*Spirogyra*	Freshwater	0.41
Ciliophora	*Heliophrya*	Freshwater	0.40
Diatomea	*Amphora*	Brackish	0.31
Diatomea	*Pleurosigma*	Brackish	0.27
Ciliophora	*Prorodon*	Freshwater	0.27
Diatomea	*Gyrosigma*	Brackish	0.25
Diatomea	*Stenopterobia*	Freshwater	0.22

We investigate the functional diversity of protists in the two environments, expressed in relative abundance of consumers, phototrophics and parasites (Fig. 1.5). Of the total OTUs, 848 were attributed to consumers (51.5%), 602 to phototrophics (43.3%), 103 to parasites (5.25%), and 37 OTUs (5.5%) were assigned to groups of organisms that can functionally range from phototrophs to heterotrophs, so we cannot unambiguously assign their functional roles. For statistical and graphical purposes, we considered only the 418 OTUs ≥1% abundant in the functional profile analyses. Our results showed a remarkable functional homogeneity between the two ecosystems, with non-significant differences between them according to the Nemenyi test (p-value > 0.05). Consumers dominate the richness in freshwater, corresponding to more than 50% of the OTUs in this environment, while in brackish water there is a higher richness of phototrophic protists (Table 1.2), although the relative abundance of functional groups was statistically equivalent in both environments (Fig. 1.5).

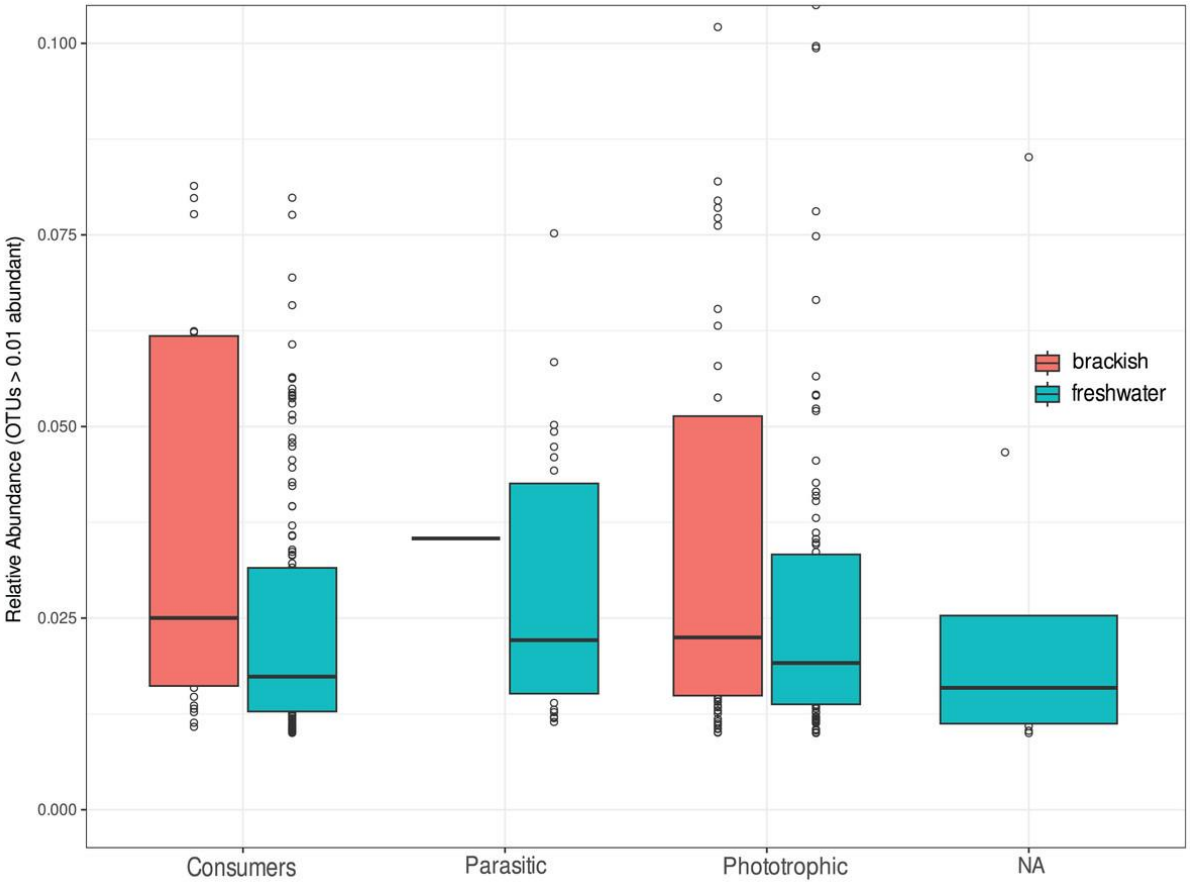

Figure 1.5 - Relative abundance of OTUs assigned to consumers, parasitic or phototrophic protists in freshwater (cyan) and brackish (coral) aquatic systems of the Brazilian Atlantic Forest. OTUs representing groups of organisms that can functionally range from phototrophs to heterotrophs are indicated as "NA" (not assigned). Relative abundances do not differ statistically by functional group of protists in these ecosystems (Nemenyi test p-value > 0.05).

Table 1.2 - Distribution of the functional diversity of protists in the Brazilian Atlantic Forest.

Functional group	Ecosystem	Number of OTUs	Corresponding %
Consumer	Freshwater	172	41.1
Consumer	Brackish	26	6.2
Parasites	Freshwater	27	6.4
Parasites	Brackish	1	1.0
Phototrophics	Freshwater	111	26.5
Phototrophics	Brackish	68	16.2
NA	Freshwater	13	3.1
NA	Brackish	0	0

1.4. DISCUSSION

1.4.1. Protist communities in coastal lagoons and freshwater systems of the Brazilian Atlantic Forest are equally diverse

The vast majority of biodiversity studies using HTS technology have been conducted in marine environments (e.g., Rychert et al., 2014; de Vargas et al., 2015; Massana et al., 2015; Gimmler et al., 2016). Relatively few metabarcoding surveys have been dedicated to investigating the diversity of inland waters, which are potentially much more diverse (e.g., Zinger, Gobet & Pommier, 2012; Balzano, Abs & Leterme, 2015; Fernandes et al., 2021). In understudied geographic regions, such as South America, these approaches are even rarer. We investigate for the first time the taxonomic and functional diversity of major protist lineages in freshwater and brackish systems located in fragments of the Brazilian Atlantic Forest. Specifically, the brackish systems studied are coastal lagoons, located in densely populated areas and considered one of the most impacted environments in the world (Esteves et al., 2008).

Our results showed that the diversity of protists in these coastal lagoons does not significantly differ from that in freshwater in terms of OTU richness and relative abundances, even though the number of samples analyzed from coastal lagoons is much smaller (six brackish vs 17 freshwater samples). This result is in contrast to Remane's concept of a minimum number of species in transitional waters (Remane, 1934), which argues that taxonomic diversity is lowest at salinities between 5 and 8 psu (Kinne, 1971). However, this concept has been shown to be based on insufficient knowledge of the taxonomic composition of organisms (Telesh et al., 2011; Telesh, Schubert & Skarlato, 2011). Conversely, bacterial and protist diversity is

usually higher in brackish waters (Telesh, Schubert & Skarlato, 2013; Santoferrara, Rubin & McManus, 2018) or comparable to other environments (e.g., Hu et al., 2016). Other diversity surveys in Brazilian coastal lagoons have reported high zooplankton diversity (Reid & Esteves, 1984; Branco, de Assis Esteves & Kozlowsky-Suzuki, 2000), with α diversity indexes comparable to that of Amazonian lakes (Carneiro, Bozelli & Esteves, 2003; Esteves et al., 2008). Here, we have observed the same pattern for protists.

Although our findings indicate that freshwater and brackish systems from Atlantic Forest are similar in terms of protist OTU richness and structure, including in relation to the functional profile of the organisms (details below), these two ecosystems differ significantly in terms of OTU taxonomic composition. Most OTUs were detected in either freshwater or brackish water, so the protist community composition differed significantly between the two environments (Fig. 1.3B). This was expected, as they are completely different ecosystems, and as previously reported for ciliates (Fernandes et al., 2021). However, a total of 23 OTUs were recorded in both ecosystems, including the bacillariophycean diatoms *Navicula*, *Amphora* and *Gomphonema*, and the ciliates *Paramecium* and *Laurentiella*, which can tolerate a wide range of salinity levels (Wilson, Cumming & Smol, 1996; Clavero et al., 2000; Smurov & Fokin, 2001).

Bray-Curtis distances were significantly greater among freshwater samples. This indicates greater heterogeneity within this sampling group in terms of OTU composition and abundance compared to brackish samples. This result was expected because the freshwater samples analyzed were taken from different water bodies, i.e., ponds, rivers, streams and waterfalls, which represent totally different environments, with different flow conditions, oxygen levels, etc. On the other hand, brackish coastal lagoons tend to be more similar to each other than to continental or marine waters, due to shared features such as strong physico-chemical gradients with adjacent ecosystems, variations in salinity and shallowness, among others (Pérez-Ruzafa et al., 2011), especially if they are geographically close and connected. Thus, these environments may share a basic set of species adapted to the same environmental conditions, or ecological guilds (Pérez-Ruzafa et al., 2011). However, due to the reduced number of brackish samples analyzed, this pattern should be considered with caution.

The freshwater samples located at Serra dos Órgãos National Park, a federal protected area (Rylands & Brandon, 2005), were the richest in protist OTUs and with the highest α diversity indexes in general. The potential of Brazilian protected areas for the discovery of new protistan taxa is underlined by the number of unclassified OTUs beyond class rank in these samples. Indeed, several new protist taxa have recently been described from the same sampling

ecosystems here investigated (e.g., Paiva et al., 2016; Campello-Nunes et al., 2015; Campello-Nunes et al., 2020; Campello-Nunes et al., 2022). This also emphasizes the importance of expanding sampling efforts in neotropical environments to enhance our comprehension of the global protist diversity.

1.4.2. Functional groups are homogeneously represented in freshwater systems of the Brazilian Atlantic Forest

Ciliates have been the richest and relatively most abundant group in the studied freshwater ecosystems. These heterotrophic organisms have a wide range of life styles and have been successful in the colonization of diverse environments (Lynn, 2008). In fact, it is one of the most represented protist groups not only in the Atlantic Forest (Simão et al., 2017; Fernandes et al., 2021), but also in other Brazilian biomes (de Araujo et al., 2018; Lentendu et al., 2019). A previous study suggested that nearly one third of the ciliate OTUs share less than 97% sequence identity with reference sequences and may represent new ciliate taxa or nominal morphotypes that have already been described, but for which 18S rRNA gene sequences have not yet been deposited in reference databases (Fernandes et al., 2021). However, heterogeneity in rRNA copy numbers in ciliate macronuclei may overestimate their relative abundances (Gong et al., 2013; Geisen et al., 2015). The second most represented group overall and the only group with higher OTU richness and relative abundance in brackish water was Diatomea, mostly the photosynthetic Bacillariophyceae, also following previous surveys in estuaries and coastal lagoons (Roselli et al., 2013; Carstensen, Klais & Cloern, 2015; Leruste et al., 2019; Stefanidou et al., 2020). This success can be attributed to the ability of these organisms to adapt to the severe environmental fluctuations inherent to transitional environments (Snoeijs & Weckström, 2010).

Regarding the functional profile of protist communities, we detected a remarkable functional homogeneity between freshwater and brackish ecosystems, with non-significant differences between them in terms of relative abundances (Fig. 1.5). This means that there is no dominance of a specific functional group, with the proportions of consumers, phototrophics and parasites roughly balanced in the investigated freshwater environments. The same applies for the investigated brackish systems, in which the proportions of heterotrophic and phototrophic protists are equivalent. However, only a single OTU classified as an apicomplexan parasite was detected, revealing low richness and abundance of protist parasites in brackish environments of the Atlantic Forest. Apicomplexa is an extremely diverse group and usually

occur in high abundances in a variety of environments, including soils, most commonly infecting metazoans (Geisen et al., 2015; Mahé et al., 2017).

Heterotrophs protists contributed more to freshwater richness than phototrophs, contrary to previous studies (e.g., Singer et al., 2021; Garner et al., 2022). In marine waters there is also a predominance of consumers, as detected by the TARA Oceans expedition (de Vargas et al., 2015). In fact, heterotrophic protists act as primary consumers, transferring significant amounts of bacterial production to higher trophic levels, contributing to nutrient cycling in aquatic food webs (Azam et al., 1983), therefore are essential components of planktonic communities in aquatic systems in general (Nagata, 1986; Jürgens & Massana, 2008). However, we detected a higher richness of phototrophic protists in brackish systems compared to other trophic groups, suggesting a protagonist of microbial photosynthesis in this ecosystem. The functional roles of protists have been extensively studied in marine waters (e.g., Caron et al., 2012, 2017), and comparatively less investigated in continental environments, such as soils and freshwater (Geisen et al., 2018; Singer et al., 2021). Investigating the taxonomic and functional diversity of protists is essential to better understand the evolution, geographic distribution patterns, and ecological roles of these organisms in the Neotropics (Ritter et al., 2021), besides being the starting point for the development of public policies for sustainability and environmental protection. Overall, our study provides valuable information on the taxonomic and trophic profile of the protist communities from the freshwater and coastal brackish systems of the Brazilian Atlantic Forest.

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CHAPTER 2

EXPLORING THE IMPACT OF URBAN POLLUTION ON CILIATE DIVERSITY ALONG THE SAPUCAÍ RIVER (MINAS GERAIS, BRAZIL) VIA DNA METABARCODING

2.1. INTRODUCTION

When analyzing diversity patterns, it is important to understand ecosystem dynamics [1]. Microbial organisms represent the majority of Earth's diversity, with protists being particularly significant [2]. The ciliates are among the most investigated protist groups due to their abundance and importance [3]. They play a crucial role in trophic networks in aquatic environments as they prey on bacteria, algae, and other microorganisms [4, 5], and can be found in a wide variety of habitats [6, 7, 8]. Additionally, they are frequently used as biological indicators due to their high environmental sensitivity, functional importance, wide distribution and abundance, small size, and short generation time [9, 10]. Nevertheless, microbial richness is likely underestimated, and much of its diversity remains unknown [11], especially in Neotropical regions [12, 13].

Biodiversity patterns in neotropics are well known for animals and plants. However, the same is not true for microbial diversity [13-15]. The molecular diversity of protists has been increasingly investigated in Brazil, especially after the popularization of high-throughput sequencing methods [8, 12, 13, 16-20]. Specifically for the phylum Ciliophora, the studies by Simão et al. (2017) [8] on the molecular diversity of ciliates in bromeliad tank waters and by Fernandes et al. (2021) [12] on the diversity of ciliates in freshwater and brackish environments in sections of the Brazilian Atlantic Forest, a biome considered a biodiversity hotspot [21], stand out. However, this biome is suffering significant impact from deforestation and pollution of water bodies [22] and still lacks broader sampling coverage in biogeographic studies [13].

Located in the Atlantic Forest, the Sapucaí River is a sub-basin of the Sapucaí River basin, encompassing the states of Minas Gerais and São Paulo in the southeastern region of Brazil. This river originates in the Mantiqueira Mountains in the city of Campos do Jordão and flows into Furnas Lake, covering 248 km and passing through several cities. It is the main watercourse supplying the municipality of Itajubá [23], an important city in southern Minas Gerais due to its industrial and educational park. The city’s development occurred around the Sapucaí River, with the central area becoming more densely populated, including along the riverbanks, due to residences, commerce, and a highway [24]. The water quality of the Sapucaí River section passing through Itajubá is concerning, since the IQA classification as "good" and "regular",

based on physico-chemical parameters, suggests that urbanization has contributed its degradation [25]. However, physico-chemical estimates of pollution levels are more meaningful when combined with biomonitoring studies [26-29]. A previous study [30] identified 48 ciliate morphospecies from a stream which flows into the Sapucaí River, being the first and only protist diversity study published so far for this region.

Considering the essential role of biodiversity studies in evaluating anthropogenic impacts and raising awareness [31], this research delves into the effects of organic pollution gradients in the Sapucaí River on ciliate communities using DNA metabarcoding. Samples of water and sediment were collected at strategic locations along the river, representing different degrees of eutrophication, to assess the influence of urban pollution on ciliate community richness, abundance, and diversity indices. Correlations between physico-chemical parameters and diversity metrics were examined to understand their impact on these communities' dynamics.

2.2. MATERIAL AND METHODS

2.2.1. Sampling and DNA extraction

In September 2022, freshwater samples were obtained from seven points along the Sapucaí River, from Piranguinho to Wenceslau Braz cities, passing through Itajubá, Minas Gerais State, Brazil. Two of these points are located downstream of the urban perimeter of Itajubá, named as Piranguinho (Pir) and Frivasa (Friv); two within the municipality of Itajubá, named CentroITJ (Itj) and PonteCabel (Cab); and three upstream, before Wenceslau Braz, named PonteDelf (Delf), PonteWB (PWB), and BambWb (BWB).

Five 200 mL aliquots of resuspended water were collected from each sampling point and stored in sterilized plastic containers, making up a total volume of 1000 mL. Additionally, values for temperature (°C), potential of hydrogen (pH), electrical conductivity (EC), total dissolved solids (TDS), and oxidation-reduction potential (ORP) of the water body were recorded at each sampling point using a digital multimeter, in order to investigate the pollution gradient along the river based on these physico-chemical parameters.

The samples were stored at room temperature overnight, then subjected to vacuum filtration using 0.22 µm polyethersulfone (PES) membranes. Total DNA extraction from the material retained on the membranes was performed using the DNeasy® PowerSoil® kit from Qiagen (Hilden, Germany), following the manufacturer’s protocol. A control sample using distilled water was also processed identically at each step to check for contamination.

2.2.2. High-throughput sequencing (HTS) and quality control of sequences

Sample preparation for Polymerase Chain Reaction (PCR), library preparation, and genetic sequencing were carried out by GenOne (https://www.genone.com.br/), as detailed in previous studies [12, 19] and briefly described here: the hypervariable V4 region of the small ribosomal subunit 18S rRNA was amplified using universal primers 528F (5′-GCG GTA ATT CCA GCT CCA A-3′) and 706R (5′-AAT CCR AGA ATT TCA CCT CT-3′) [32, 33]. The V4 region was chosen because it has been demonstrated that its phylogenetic signal corresponds to that of the complete eukaryotic 18S rRNA gene for ciliates [34]. The concentration and quality of the amplified DNA were monitored via 2% agarose gel electrophoresis and Qubit® spectrophotometer. The libraries were sequenced on Illumina MiSeq to generate 250 bp paired-reads. The obtained sequences were processed and assembled using FLASH V1.2.7 (http://ccb.jhu.edu/software/FLASH).

The reads were demultiplexed and quality-filtered using QIIME2 [35]. First, quality control was performed using the *q2-dada2* plugin, removing chimeric and low-quality reads (*q-score* < 25). To eliminate possible artifactual sequences, reads with a frequency less than 10 were also removed [36]. Then, using the *q2-feature-table* plugin, we obtained a feature table including data from high-quality reads. For obtaining operational taxonomic units (OTUs), a 97% identity threshold was adopted, as proposed in previous studies [12, 37], using the *q2-vsearch* plugin and the open-reference method [38] against the SILVA database version 138 [39]. The sklearn classifier pre-trained on SILVA 138 was used for OTU taxonomic annotation [40] using the *q2-feature-classifier* plugin [41]. Lastly, the OTUs assigned to the Phylum Ciliophora were extracted for diversity and phylogenetic analysis with the phyloseq and vegan R-packages [42, 43].

2.2.3. Diversity analyses

To estimate richness, composition, and alpha-diversity indexes for ciliates in the samples, the number of unique observed OTUs, Shannon's diversity index H’ [44], and Simpson's diversity index D [45] were considered using the *diversity* function of the phyloseq R-package. The OTU richness in the samples was also estimated using species accumulation curves through the *specaccum* function of the vegan R-package [43]. For beta-diversity analyses, the samples were categorized based on their distribution along this stretch of the river in downstream (Pir, Friv), urban area (Itj, Cab) and upstream (Delf, PWB, BWB) (Table 2.1). The physico-chemical parameters mentioned were recorded during sampling to explore potential correlations with the

observed diversity in the samples. Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarities, unweighted (qualitative) and weighted (quantitative) UniFrac distances were used, using the *vegdist* function of the vegan. The significance of differences between samples was assessed using the Permanova test by the *adonis* function. The NMDS (Non-metric Multidimensional Scaling) plot of Bray-Curtis dissimilarities was generated using the *envfit* function to evaluate the impact of physico-chemical parameters on ciliate diversity across the pollution gradient.

Table 2.1 - Identification, richness and alpha-diversity metrics by sampling site.

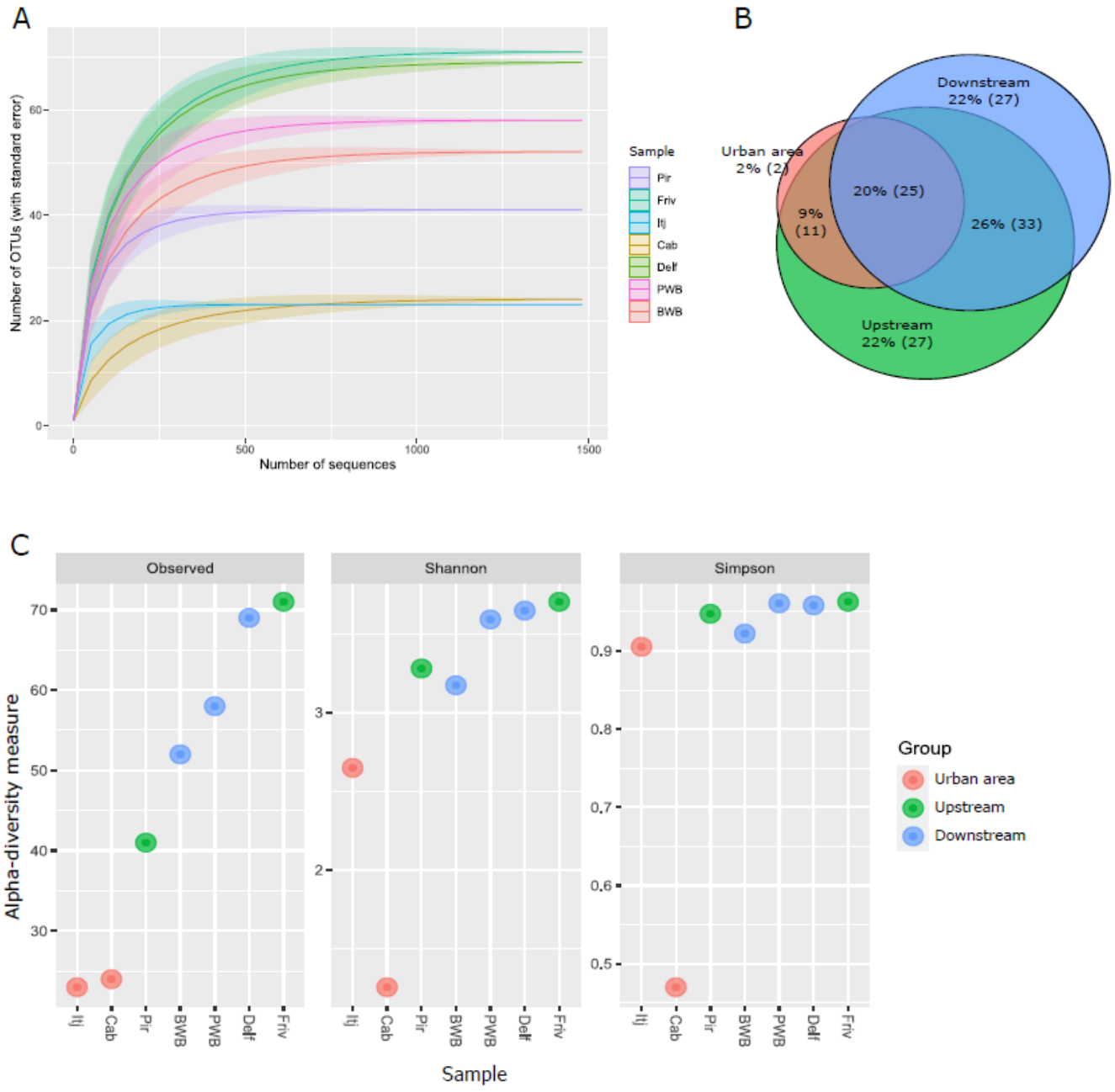
Sample ID	Group	Abb	Number of ciliate OTUs	Shannon's index H	Simpson's index D
Piranguinho	Downstream	Pir	41	3.28	0.95
Frivasa	Downstream	Friv	71	3.70	0.96
CentroITJ	Urban area	Itj	23	2.65	0.91
PonteCabel	Urban area	Cab	24	1.26	0.47
PonteDelf	Upstream	Delf	69	3.65	0.96
PonteWB	Upstream	PWB	58	3.59	0.96
BambWB	Upstream	BWB	52	3.17	0.92

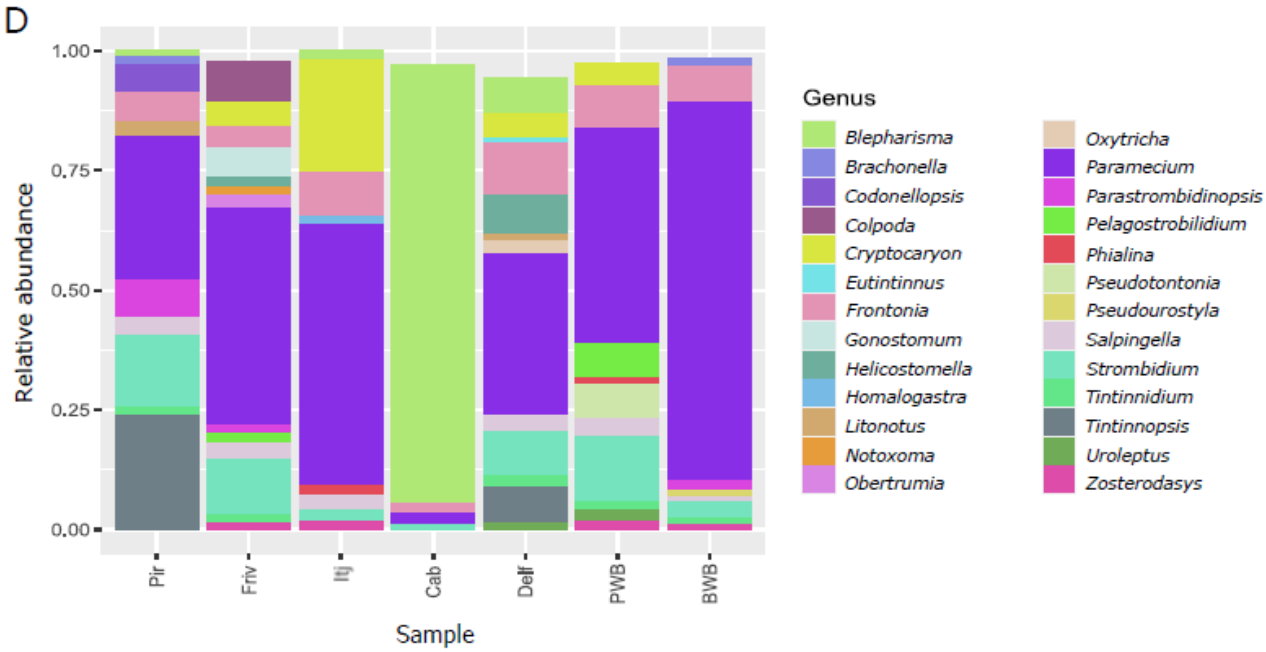
2.3. RESULTS

From the sequencing of the samples, a total of 482,330 reads were obtained. Following quality control, 375,062 reads with an average length of 220 bp were retained. These sequences were then clustered into 993 operational taxonomic units (OTUs) of eukaryotes, of which 125 OTUs were identified as Ciliophora.

Analyzing the species accumulation curves, all samples reached the saturation plateau, indicating that the entire diversity could be recovered from the sampling (Fig. 2.1a). The upstream samples from Itajubá had a total of 96 OTUs, and the downstream samples had 85 OTUs. Both showed greater richness compared to the samples from the urban area, which contained only 38 OTUs (Fig. 2.1b). The richest samples were Friv (71 OTUs) and Delf (69 OTUs), located downstream and upstream, respectively. The samples with the lowest richness were Itj (23 OTUs) and Cab (24 OTUs), both from the Urban area. The Simpson and Shannon diversity indices were also higher in the upstream and downstream samples (Fig. 2.1c). Notably, the Cab sample from the urban area showed slightly lower values in the alpha diversity analysis (Table 2.1).

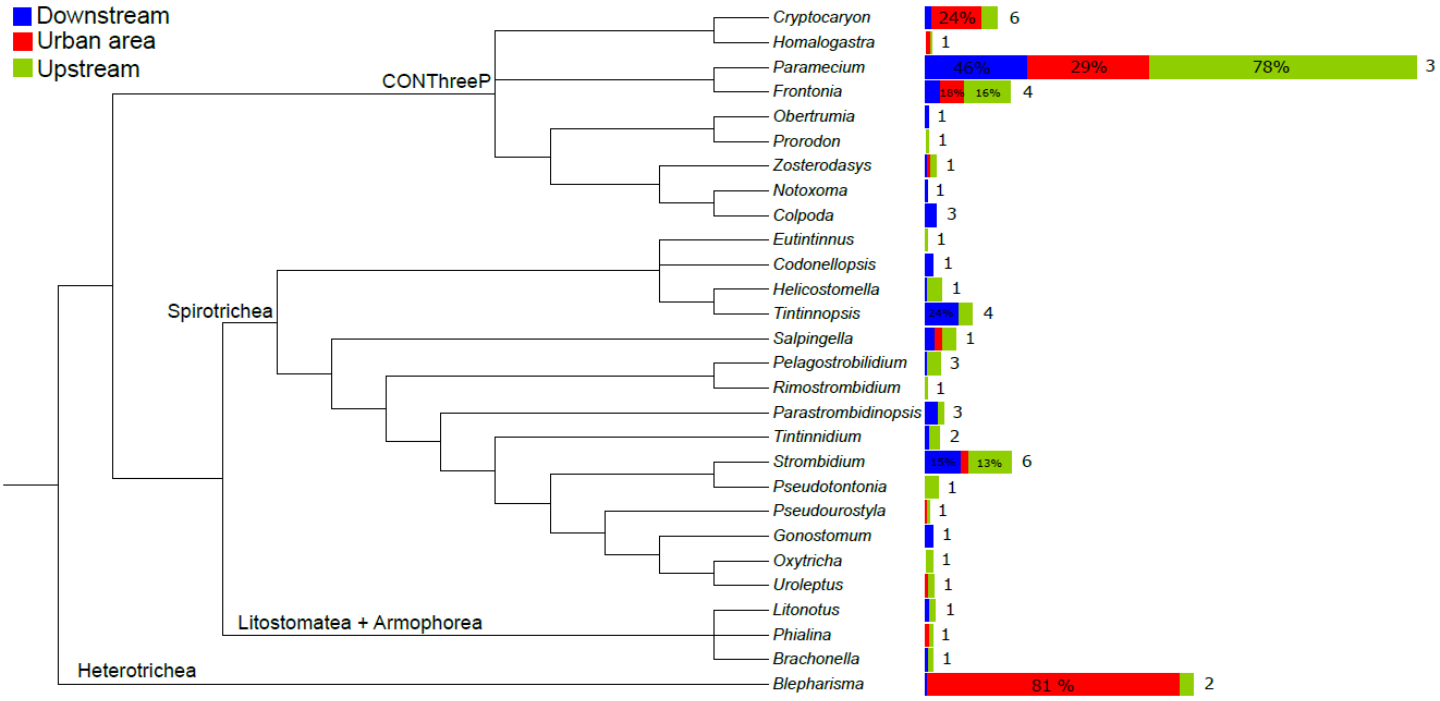
Figure 2.1 - Richness and diversity of protist ciliates from Sapucaí River, Minas Gerais State, Brazil. **A** Species accumulation curves based on the number of Operational Taxonomic Units (OTUs) per sample. **B** A Venn diagram illustrating the distribution of OTUs across the sampling groups — downstream, urban area and upstream — depicting both unique and shared OTUs among the groups. **C** Number of observed OTUs, the Shannon and Simpson diversity indices calculated for each sample within every group. **D** Relative abundance of ciliate genera per sample.





Of the 125 identified OTUs, 123 (98.4%) belong to the subphylum Intramacronucleata and only two OTUs (1.6%) belong to the subphylum Postciliodesmatophora. Out of the total, 65 OTUs were classified as belonging to the class Spirotrichea, 14 to Litostomatea, 2 to Armophorea, and 2 to Heterotrichea. A total of 42 OTUs were classified as CONThreeP, a clade derived from the last common ancestor of Colpodea, Oligohymenophorea, Nassophorea, Phyllopharyngea, Prostomatea, and Plagiopylea [46]. At the genus level were identified 54 OTUs, representing 28 genera, with *Cryptocaryon*, *Strombidium* (6 OTUs each), and *Frontonia* (4 OTUs) being the most representative, followed by *Colpoda*, *Paramecium*, *Parastrombidinopsis*, and *Pelagostrobilidium* (3 OTUs each) and *Blepharisma* and *Tintinnidium* (2 OTUs each). The remaining 22 OTUs were classified into 22 different genera (Fig. 2.2). Finally, beyond the genus level, 71 OTUs could not be identified.

Figure 2.2 - Schematic phylogenetic tree of identified ciliate genera and their respective relative abundances per sampling group: downstream (blue), urban area (red) and upstream (green). Following the bars, the count of observed OTUs categorized by genus.



Only the genera *Frontonia*, *Strombidium*, and *Paramecium* had relative abundance ≥1% in all samples (Fig. 2.1d). The vast majority of the genera had low relative abundance. The genus *Frontonia* showed 18% relative abundance in the Itj sample (urban area) and 16% in the PWB sample (upstream). Ciliates of the genus *Strombidium* represented 15% of the Pir sample (downstream) and 13% of the PWB sample (upstream). The genus *Cryptocarium* accounted for 24% of the relative abundance in the Itj sample (urban area) and *Tintinnopsis* for 24% in the Pir sample (downstream). The genus *Paramecium* was considerably the most abundant in all samples, except for the Cab sample (urban area), which was almost entirely dominated by *Blepharisma* (81%).

Beta-diversity was assessed using Bray-Curtis dissimilarity indices with the *permutest* function (*p-value* = 0.001) and *anova* function (*p-value* = 0.008), concluding that the three sampling groups (downstream, urban area, and upstream) differ significantly in terms of ciliate community composition (Fig. 2.3). Additionally, the adonis test revealed that this distinction is independent of the physico-chemical characteristics of the environment (Table 2.2), indicating that these parameters are not associated with higher or lower ciliate diversity in the samples (Fig. 2.4; *p-value* > 0.05).

Figure 2.3 - Principal Coordinates Analysis (PCoA) with Bray-Curtis distances to visualize the dissimilarities in ciliate community composition across sampling groups. The Permanova test revealed significant differences in ciliate diversity among downstream, urban area, and upstream samples.

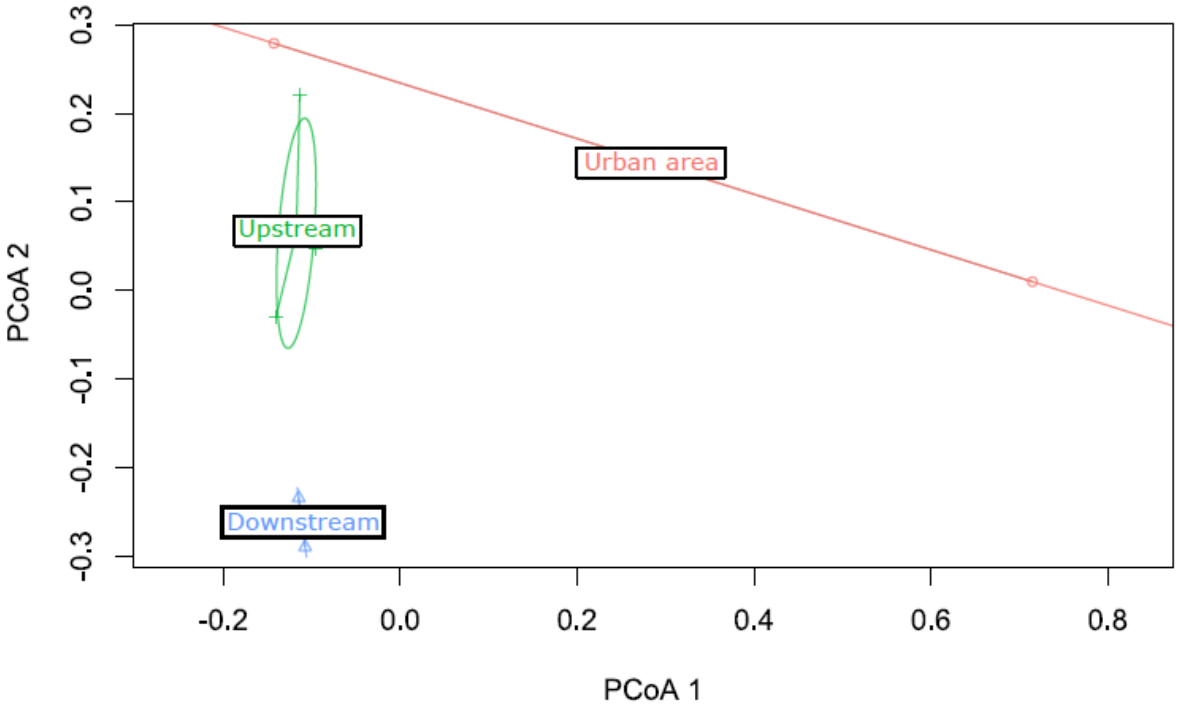
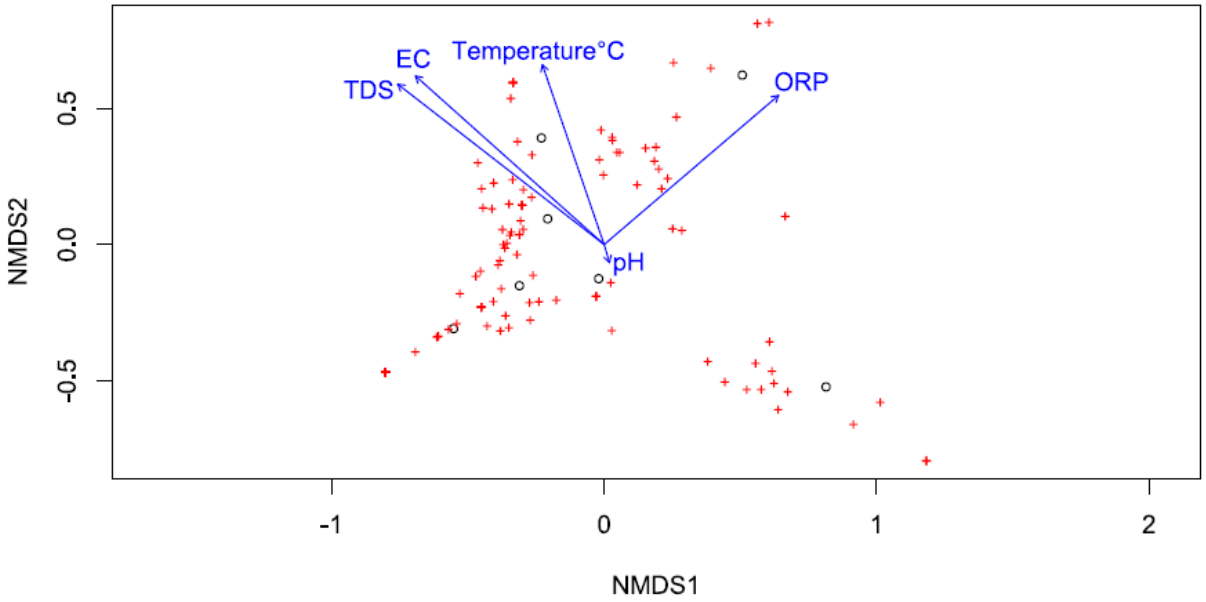


Table 2.2 – Physico-chemical parameters recorded for each sample. pH = potential of hydrogen; EC = electrical conductivity; TDS = total dissolved solids; ORP = oxidation-reduction potential.

Sample ID	Temperature (°C)	pH	EC (µS/cm)	TDS (ppm)	ORP (mV)
Piranguinho	19.3	7.03	70	35	78
Frivasa	18.8	6.96	56	28	64
CentroITJ	18.6	7.16	49	24	88
PonteCabel	18.2	7.19	44	22	83
PonteDelf	17.7	7.33	49	25	47
PonteWB	18.6	7.61	54	28	91
BambWB	18.2	7.48	55	27	79

Figure 2.4 - Non-metric Multidimensional Scaling (NMDS) based on Bray-Curtis distances. The position and length of arrows in relation to the centroid of the samples (white circles) suggest that the variations in ciliate communities are not correlated with the recorded physico-chemical parameters.



2.4. DISCUSSION

According to the analysis of ciliate communities, the samples from the stretch of the Sapucaí River located in the urban perimeter of Itajubá (urban area) showed lower richness compared to the upstream and downstream samples. The same was observed for the Shannon and Simpson diversity indexes, which were lower for samples from urban area (Fig. 2.1). This stretch of the river receives a higher discharge of organic pollutants from regions that still lack a sewage treatment system, as well as from some of its tributaries [24, 47]. Additionally, this watercourse is affected by urbanization, leading to possible hydrographic changes, increased concentrations of nutrients and contaminants [48]. These characteristics affect the diversity of ciliate protists, as they are microorganisms highly sensitive to pollution and they have a shorter response time to environmental variations due to their rapid generation time [9, 10]. Analyzing the diversity of the samples, the class Spirotrichea was the most abundant, followed by Oligohymenophorea and Litostomatea with 65, 19, and 14 OTUs, respectively, representing almost 80% of the total. These classes, besides being quite diverse, are frequently found in studies of freshwater ciliates in different regions and biomes [12, 18, 20, 30, 49, 50]. The genera *Strombidium*, *Frontonia*, and *Paramecium* were the only ones present in all samples (Fig. 2.2). Ciliates of the genera *Frontonia* and *Paramecium* are commonly found in freshwater environments with considerable abundance [12, 51, 52]. However, ciliates of the genus

Strombidium is more commonly found in marine environments, but also has freshwater representatives, mainly in lakes. It is a versatile organism with a mixotrophic metabolism, able to feed on algae and bacteria and sequester plastids from its prey [53-55]. They are generally planktonic and, due to their feeding strategies, are found in oligotrophic environments with low nutrient availability, and they can exhibit tolerance to stressful conditions [56]. Its persistence in environmental changes and versatility in nutritional strategies may explain its presence in all studied samples, as well as observed before [12].

The genera *Litonotus*, *Blepharisma*, *Paramecium*, *Frontonia*, and *Dileptus* were observed both in the current study and in a previous investigation of the morphological diversity of ciliates in the José Pereira stream [30], a tributary of the Sapucaí River that lacks sewage treatment. These genera are included in the saprobic system [57], with most indicating polluted water conditions. Notably, the genus *Blepharisma* was dominant in the Cab sample (urban area), presenting 81% of relative abundance. Its dominance may be possibly correlated to the production of blepharismin, a toxic pigment capable of inhibiting the growth of other cells [58] and, therefore, other organisms present in the habitat, contributing to the decreased richness of this sample. The genera *Paramecium* and *Frontonia* exhibited the highest relative abundances. They are typically associated with environments ranging from moderately to highly polluted [9, 57, 59-62] and can be found in various types of water bodies, sediments, and even soil [60-62]. The notable presence of these ciliates in the analyzed samples indicates that they are key components of these microeukaryote communities.

For the appropriate application of the saprobic index according to Foissner [57], organisms need to be identified at the species level and have well-studied ecology and phylogeny [63]. Among the 28 OTUs identified to the species rank in the present study, only three are on Foissner's list and have known saprobic indices, with *Lembadion bullinum* being beta-mesosaprobic, i.e, present in slightly polluted environments, moderate organic load, and slight oxygen deficit), *Paramecium caudatum* being alpha-mesosaprobic (polluted environments, high organic matter content, and reduced dissolved oxygen levels), and *Acineria incurvata* being polysaprobic (highly polluted environments, with a high organic load and very low or no dissolved oxygen levels). These three species were observed in the three sample groups.

The vast number of OTUs that could not be classified to species rank (more than 75%) or at least to the taxonomic category of genus (more than 55%) reflects the lack of reference sequences in the current molecular databases. This is not only because microbial richness is underestimated in neotropical environments, but also due to the challenges in species

identification and the insufficient investment in taxonomic studies [11, 13, 21, 63]. The metabarcoding approach for biodiversity monitoring has become a promising alternative to traditional morphological methods [63, 64]. However, its use has some considerable limitations. OTUs correspond to a grouping of sequences based on a percentage of similarity and may not necessarily correspond to a biological species [65]. Furthermore, species identification is only possible if reliable reference sequences have already been deposited in a database [66]. Besides the existing gaps, the lack of curation of databases can also lead to inaccuracies in taxonomic identifications [67]. Thus, it is crucial to stress the importance of combining both morphological and molecular methods in diversity studies and environmental assessments [68].

The analysis of ciliate richness and diversity in the investigated stretch of the Sapucaí River confirmed the influence of the pollution gradient on these communities. The composition of ciliates differed significantly among the samples obtained upstream, in the urban area, and downstream of Itajubá city, with the urban area being the least diverse. Other studies have shown similar results, with ciliate communities varying significantly along water bodies due to organic pollution and human activities [69-71]. The physico-chemical parameters showed little variation between the samples and were not related to the changes observed in the ciliate communities along the river. Our research demonstrated that ciliate protists are strongly affected by environmental changes and respond more sensitively to these disturbances than physico-chemical parameters, supporting previous findings [72-74]. Hence, employing them as bioindicators is crucial for environmental quality assessments.

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CONCLUSION

Given the importance of protists as key components of ecosystems and their ubiquitous presence in various habitats, studying their diversity and functional roles in these environments goes beyond filling taxonomic knowledge gaps; it also allows for the improvement of public policies aimed at environmental sustainability. However, these organisms are still very poorly understood, especially in neotropical regions. In the samples studied, protist communities in coastal lagoons and freshwater systems of the Atlantic Forest differ significantly in terms of taxonomic composition, as expected. Nonetheless, they are equally diverse in terms of richness and functional profile, contrasting with the minimal number of known species in transitional waters and highlighting the insufficient understanding of the taxonomic composition of these organisms. Despite most biodiversity studies being conducted in marine environments, freshwater samples exhibited greater heterogeneity in protist community composition. This underscores the importance of expanding investigations in inland waters.

Studies of protist community composition indicate that ciliates are the richest and most abundant group in freshwater ecosystems, and this study was no exception. In addition to species richness and functional importance, this group has high environmental sensitivity and potential as bioindicators. Evaluating ciliate diversity in different sections of the Sapucaí River (Itajubá-MG, Brazil) corroborated the impact of anthropogenic actions on these communities. The samples from the urban area had the lowest richness and diversity, with the three sampling groups (Upstream, Center, and Downstream) differing significantly. Since the physico-chemical parameters showed little variation among the samples, ciliates were strongly affected by environmental changes, responding more sensitively to these disturbances than physico-chemical parameters.

Beyond providing valuable information on the functional and trophic profiles of protist communities in freshwater and coastal brackish systems of the Brazilian Atlantic Forest, it was possible to highlight the impact of anthropogenic actions on ciliate protist communities in a river with different eutrophication gradients and the potential of these organisms as bioindicators. The importance of conserving microbiota and understanding the ecosystems they inhabit becomes essential for sustainable development.

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