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ECOSYSTEMS

Diversity of eukaryotic and prokaryotic microbiota revealed by metabarcoding in Neotropical floodplain lakes

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Abstract: The diversity of eukaryotic and prokaryotic communities has been assessed by morphological and genetic approaches, which are used to characterize the microbiota in different environments. Here, planktonic prokaryotic and eukaryotic communities of the Araguaia River, located in the Central region of Brazil, were analyzed based on metabarcoding analysis of rRNA genes to evaluate the diversity of these groups in tropical floodplain lakes. Also, we tested their spatial concordance throughout the Araguaia river. Water samples were collected from 8 floodplain lakes in Araguaia River. The 16S and 18S rRNA genes were amplified and sequenced using Illumina MiSeq. For eukaryotes, 34,242 merged reads were obtained and 225 distinct OTUs were delineated, of which 106 OTUs were taxonomically classified. For prokaryotes, 26,426 sequences were obtained and 351 OTUs were detected. Of them, 231 were classified in at least one taxonomic category. The most representative eukaryotes belonged to Ciliophora, Chlorophyta and Charophyta. The prokaryotic phylum with the most OTUs classified were Proteobacteria, Actinobacteria and Bacteroidetes. The lakes did not show spatial concordance when comparing the similarity between their microbiota. The knowledge of freshwater biodiversity using DNA sequencing for important rivers, such as Araguaia River, can improve microbiota inventories of tropical biodiversity hotspots.

Key words: Araguaia river, aquatic community, environmental DNA, freshwater, genetic biodiversity.

INTRODUCTION

Freshwater environments have a high biodiversity, with a large number of endemic species; however, these environments are among the most threatened on the planet (Dudgeon et al. 2006, Strayer & Dudgeon 2010, Irvine et al. 2016). The main impacts may include habitat degradation, water pollution, structural changes with altered flow, insertion of exotic species as well as overexploitation of these environments and interactions between these different factors (Dudgeon et al. 2006). Thus, the identification of complete biodiversity of freshwater tropical communities may be a powerful resource for ecology and conservation of these regions.

In Brazil, the Araguaia River is located in Tocantins-Araguaia basin, and represent one of the most important drainage channels in Central region of Brazil (Valente et al. 2013). It extends all the way crossing the Cerrado, the second major Brazilian Ecosystem, and is considered a hotspot of biodiversity (Myers et al. 2000). Along the Araguaia River, there are important changes on ecosystems and land usage. Recently, some studies have explored

the microbiota of freshwater ecosystems, including tropical environment, through genetic approaches (Arroyo et al. 2018, Cahoon et al. 2018, Li et al. 2018, Santos-Júnior et al. 2020). However, for the Araguaia River, few studies have used this strategy to investigate the microbiota composition (e.g. Tessler et al. 2017, Lentendu et al. 2019, Machado et al. 2019), and do not consider the prokaryotic and eukaryotic fraction of the microbion community simultaneously (see Tessler et al. 2017 for bacteria and Lentendu et al. 2019 and Machado et al. 2019 for protists).

Bacteria and microeukaryotes play an important role in aquatic ecosystems, acting on biogeochemical cycles and establishing interactions with different levels of the food web (Cotner & Biddanda 2002). Furthermore, microbiota composition may indicate the conditions of the environment, as the trophic state or also the presence of disturbance factors (Baird & Hajibabaei 2012, Dowle et al. 2016, Monteagudo & Moreno 2016, Röhl et al. 2017). It is known that the diversity of microorganisms on earth is very high, however, it has not yet been possible to reach a consensus on this exact value (Dunlap 2001), including, the aquatic microbiota. Thus, the metabarcoding approach appears to be faster and more informative than morphology-based methods to assess species richness in natural communities, as well as more accurate, providing increased taxonomic resolution (Baird & Hajibabaei 2012, Bradford et al. 2013, Stein et al. 2014).

In metabarcoding approach, environmental DNA (eDNA) from whole communities is extracted from samples such as water and soil. Hypervariable subregions of the 16S and 18S ribosomal RNA (rRNA) genes are sequenced using high-throughput sequencing technologies (Coissac et al. 2012, Yang et al. 2016) and used to identify and classify prokaryotic and eukaryotic organisms, respectively, to determine taxonomic units in different ecosystems (Nam et al. 2012, Fouts et al. 2012, Baldwin et al. 2013, Klindworth et al. 2013, Hadziavdic et al. 2014, Yang et al. 2016, Nia et al. 2017). Metabarcoding studies in freshwater aquatic ecosystems can be used to evidence changes in microbiota communities as well as to correlate the presence of prokaryotes and eukaryotes in specific sites or time (Bock et al. 2018).

Herein the diversity of eukaryotic and prokaryotic microbiota of important floodplains lakes in Central Brazil was described based on metabarcoding analysis of rRNA genes. Also, the spatial concordance of the lakes using two metabarcoding analysis (16S and 18S rRNA genes) was analyzed. New information was generated to characterize the diversity of organisms of this important Brazilian freshwater system that complemented the information produced by traditional taxonomic studies in this region. In addition, this study contributes to a better understanding of the composition of aquatic microbiota in the tropics.

Abbreviations

eDNA – environmental DNA
rRNA – Ribosomal RNA
bp – base pairs
OTU – operational taxonomic unit
PCA – Principal Componet Analysis
UPGMA - Unweighted Pair Group Method using Arithmetic Averages
rDNA – ribosomal genes

MATERIALS AND METHODS

Study area

We investigated 8 floodplain lakes (13° 26’ 2.79”S to 11° 47’ 15’’ S and 50° 43’ 38.3988’’ W to 50° 32’ 3.4008’’ W), located along of the middle course of Araguaia River (Lakes 1 – 7) and one of its tributaries (Mortes river, Lake 8) during

the high-water season in January 2012 (Figure 1). These lakes are shallow, oligotrophic and with warm waters (see details in Machado et al. (2015) and Marcionilio et al. (2016)). Most lakes are still surrounded by Cerrado native vegetation (Machado et al. 2016).

Samples

In each lake, we measured limnological parameters of water, including the temperature, transparence, turbidity, oxygen saturation, dissolved oxygen, pH, depth, conductivity and total dissolved solids. The estimative of chlorophyll-a, total nitrogen and total phosphorus were performed according to Golterman et al. (1978) and Zagatto et al. (1981). For each lake, we also estimated the surrounding land cover (pasture, agriculture, native Cerrado vegetation), and morphometrics parameters as area and width. The full description of the methods for morphometrics and land cover data has been described previously in Machado et al. (2016) and Marcionilio et al. (2016).

Water samples for eukaryotic communities were obtained by filtration of 500 liters of water through a plankton net (68 µm mesh aperture) using a vacuum pump. The sampling was performed in the central region of each lake, that is, in the same region where the parameters of water were evaluated. Thus, we obtained an individualized sampling for each floodplain lake, independent of each other. The concentrated sample resulting from this first filtration (250 mL water) was filtered on a Millipore cellulose filters (3.0 µm pore size), considering the separate samples for each of the eight lakes. For prokaryotic communities, 500 mL of water were collected directly in each lake and filtering performed separately for samples from each lake, firstly, using a Millipore cellulose filter (3.0 µm pore size) and then further filtered using a Millipore cellulose filter of 0.22 µm pore size, which was used for evaluation of the prokaryotic communities. The filters for prokaryotic and eukaryotic microbiota were stored in liquid

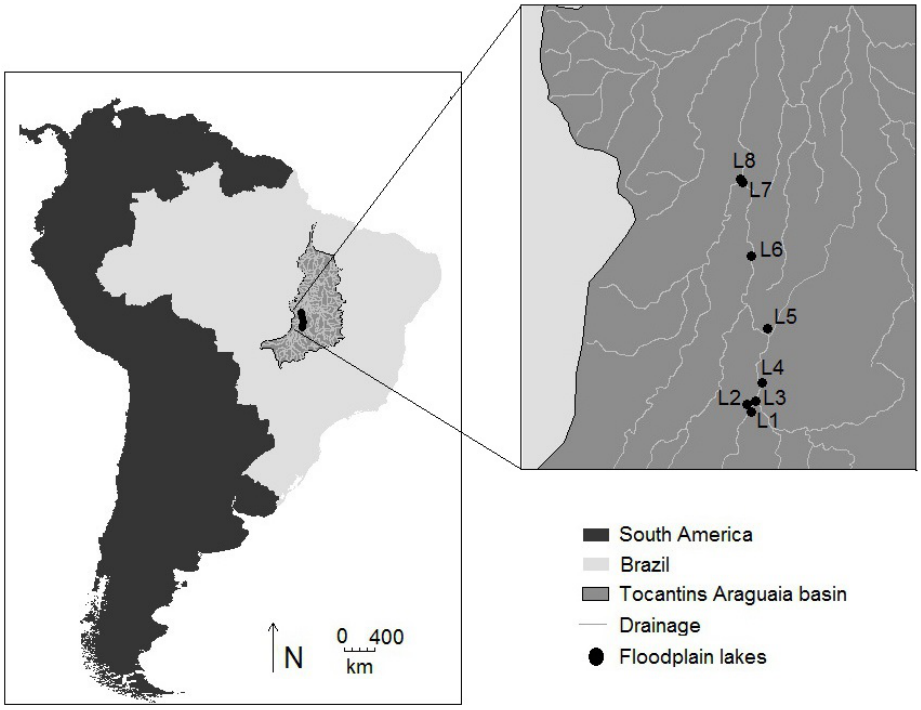

Figure 1. Sampled locations. Lakes sampled along the Araguaia River floodplain, Brazil.

nitrogen at -80 ° C separately each other and separately between the eight lakes.

eDNA preparation and Taxonomic assignment

Genomic DNA was extracted from filters using PowerWater DNA Isolation kit (Mo Bio) from each site collected. For eukaryotes, the V4 region from 18S ribosomal DNA region was amplified by PCR using the universal primers TAReukFWD1 and TAReukREV3 (Stoeck et al. 2010) modified with complementary sequences containing Illumina Adapters (Illumina). For prokaryotes, the 16S ribosomal DNA was amplified using BAC341F and BAC785R (based on Klindworth et al. 2013) also with complementary sequences Illumina Adapters (Illumina). The PCRs amplifications followed the reaction published in Machado et al. (2019) and were done in triplicates for each sample site. The PCR samples were pooled and amplicons were used to prepare the NGS libraries as described also in Machado et al. (2019). Amplicons libraries were pooled and sequenced using MiSeq Reagent Kit v3 (600 cycles) on MiSeq platform (Illumina) installed at the Genetics and Biodiversity Laboratory, Federal University of Goiás, state of Goiás, Brazil.

The quality of the sequences was evaluated by FastQC software (Andrews 2010) and they were trimmed using Trimmomatic (Bolger et al. 2014). Bases with phred < 20 were excluded as well as reads smaller than 100 base pairs (bp). The Illumina adapters were also removed. We used clustering methods to infer broader biological diversity in Araguaia River. We also applied rarefaction on our data to minimize inferences of method bias (see below). The OTU prediction was performed with UPARSE pipeline (Edgar 2013) for eukaryotes and prokaryotes, separately. Thus, the paired-end reads of each sample were first assembled (merged) and OTUs were delineated at 97% similarity. Taxonomic annotation was performed by Blasting representative OTU sequences against the database Silva 132 (https://www.arb-silva.de/ access in November 2019) using a percentage of identity > 97%. Statistical analyzes were performed with the BioEstat program version 4.0. For the consecutive analysis of the OTU diversity, we removed the ones that were classified as Metazoa, Plants and non-planktonic organisms.

The sequences were deposited in the GenBank database (https://submit.ncbi.nlm.nih.gov/) under the access number SUB7351368 for Eukaryotes and SUB7351387/SUB7435447 for Prokaryotes. (Bioproject Accession PRJNA629519 and BioSample Accession SAMN14777710 - SAMN14777717 for Eukaryotes SAMN14777757 - SAMN14777764 for Prokaryotes).

Data analysis

We use a principal component analysis (PCA) seeking to compare the floodplain lakes in relation to their environmental conditions. In PCA, the limnological data were standardized by the z score method and the land cover data by arcsines of their square roots ×180/ π. The PCA was performed using the "prcomp" function of vegan package (Oksanen et al. 2017), in R Platform (R Core Team 2020).

Species accumulation curve was used to estimate whether the sampling was sufficient for Eukaryotic and Prokaryotic communities (see details in Ugland et al. 2003). A subsampling rarefaction was used to correct the bias that can be generated by comparing samples with different sizes. The rarefaction was conducted through a random subsampling, in which the sample size was represented by the lowest number of sequences recovered in floodplain lakes, that is 2,208 reads for prokaryotes and 1,649 reads for eukaryotes (Hurlbert 1971).

We used Pearson correlations to assess the relationship between the number of reads and merged sequences; and the number of merged

sequences and the total number of predicted OTUs for both prokaryotes and eukaryotes microbiota. Cluster analyses was performed to investigate the similarity between sampled point based on the occurrence of OTUs from different microbiotas (16S and 18S rDNA). The matrices were generated based on Jaccard index and the dendrogram was created by UPGMA method (Unweighted Pair Group Method using Arithmetic Averages (Legendre & Legendre 2012). The dendrograms generated for the eukaryotic and prokaryotic microbiota were visually compared using the "tanglegram" function, from the dendextend package (Galili 2015), in the R platform (R Core Team 2020).

The Mantel test was used to investigate the spatial concordance between the occurrence matrices of 16S and 18S rDNA microbiota, based in Jaccard distance. The Mantel test varies between -1 (negative concordance), to 1 (perfect positive concordance). Zero indicates no concordance among matrices. The Mantel test was performed using the "mantel" function with the *Vegan* package (Oksanen et al. 2017), in R Platform (R Core Team 2020).

RESULTS

For the environmental analysis, the first and second axis of the PCA explained 67% of the variation in environmental data. The floodplain lakes sampled in this study are environmentally heterogeneous, mainly in relation to the limnological parameters, and were grouped into two groups according to their environmental conditions (Figure 2). Lakes 1 to 4 were positively associated with turbidity, native vegetation, and negatively associated with dissolved oxygen, saturated oxygen, nitrogen and total phosphorus, pH, area and width. Lakes 5 to 8 were positively associated with temperature, transparency, conductivity, and negatively associated with agriculture, pasture, total dissolved solids, depth and chlorophyll-a.

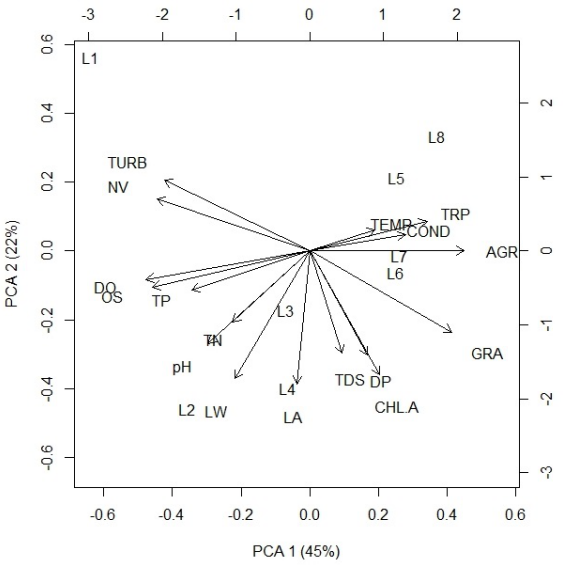

Figure 2. Environmental PCA analysis. Principal component analysis for environmental data measured in eight floodplain lakes in the middle Araguaia River. COND, conductivity; DO, dissolved oxygen; pH, potential of hydrogen; TEMP, temperature; TURB, turbidity; TDS, total dissolved solids; TRP, transparency; OS, oxygen saturation; DP, depth; TP, total phosphorus; TN, total nitrogen; CHL.A, chlorophyll-a; LA, lake area; LW, lake width; NV, native vegetation; GRA, grassland; AGR, agriculture.

In order to assess the Eukaryotic biodiversity, from the sequenced samples, it was detected 760,148 raw reads and 509,639 reads after quality control from all collected sites. From them, the total merged sequences were 34,242 with mean of 383 bp (Table I). We did not find a correlation between the number of reads (after quality cuts) and the number of sequences merged ($r = 0.19$ and $p < 0.65$). The numbers of reads and merged sequences varied along the collected sites (Supplementary Material - Figure S1a, b). From all merged sequences, it was possible to detect 225 distinct OTUs. No significant Pearson correlation was observed between the number of merged sequences and the total number of predicted OTUs ($r = 0.32$ and $p = 0.43$). Site L2 and

Table I. Comparation of eukaryotic and prokaryotic communities on Araguaia River based on metabarcoding analysis of rRNA genes data obtained at Miseq platform and subsequent bioinformatic analyses.

	Eukaryotic	Prokaryotic
Total raw reads	760,148	2,131,925
Total filtered reads	509,639	1,273,990
Merged sequences	34,242	26,426
Medium size of sequences (bp)	383 bp	403 bp
Total rarefied OTUs	206	344
OTUs taxonomic classified	106	231

L8 showed the lesser and the greatest amount of OTUs, respectively (Figure S1c). After rarefaction, the total number of OTUs was reduced to 206. Among these, 106 OTUs were taxonomically classified when compared to database.

In all sampled sites, some phyla were more diverse concerning the numbers of OTUs taxonomically classified (Figure 3). The most frequent Eukaryotic phylum was Ciliophora (26 OTUs), followed by Chlorophyta (19 OTUs) and Charophyta (15 OTUs), while groups as Centrohelida, Chytridiomycota and Chanoflagellida presenting only one OTU each. Inside the kingdom Fungi, the phylum Cryptomycota was the most diverse (three OTUs), followed by Ascomycota (two OTUs) and Chytridiomycota (one OTU). For Chromalveolata group, Ciliophora was the phylum with more different OTUs (26 units), followed by Ochrophyta (14 units).

The prokaryotic community detected from the genetic analysis had 2,131,925 raw reads that were filtered to 1,273,990 reads for all collected sites. The reads were merged into a total of 26,426 sequences with a mean size of 403 bp that were used to predict the OTUs (Table I). No significant correlation was found between the number of reads after quality control and the number of

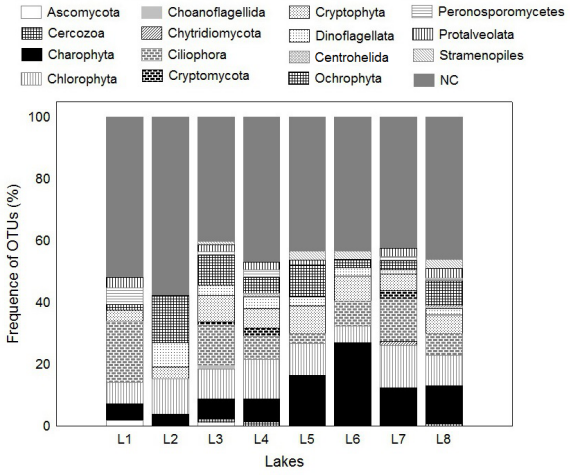

Figure 3. Frequency of Eukaryotes. Frequency of eukaryotic OTUs according to the taxonomic group in each sample point. NC = Not Classified.

sequences merged (r = 0.47 and p = 0.23). The numbers of reads and merged sequences varied along the collected sites (Figure S2a, b).

Using all merged sequences, it was possible to identify 351 OTUs. No significative correlation was observed between the number of sequences merged and the total number of predicted OTUs (r = 0.36 and p = 0.37). The sites L3 and L8 presented the major and the minor numbers of different OTUs, respectively (Figure S2c). After rarefaction, the number of prokaryotic OTUs reduced to 344, and when compared to database, 231 OTUs were at least classified in one taxonomic category. Three prokaryotic phyla had the most OTUs identified: Proteobacteria, Actinobacteria and Bacteroidetes (Figure 4). Planctomycetes was only presented in lakes L3 and L4, while representatives of Armatimonadetes was only seen in lakes L1, L3 and L4.

The rarefaction curves for both prokaryotes (Figure S3a) and eukaryotes (Figure S3b) indicate that the diversity of the microbiota was not fully covered in some of the sampled lakes. However, the accumulation curve for prokaryotes has reached a plateau (Figure S4a), indicating that the gamma diversity for this portion of

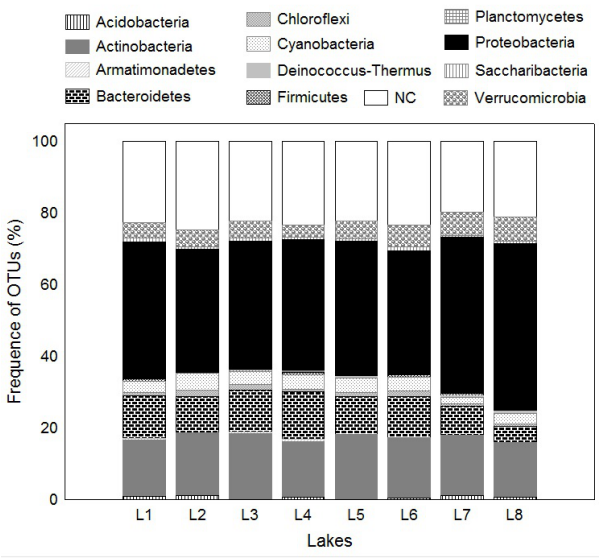

Figure 4. Frequency of Prokaryotes. Frequency of prokaryotic OTUs according to the taxonomic groupin each sample point. NC = Not Classified.

the microbiota has been fully covered in the region. For eukaryotes, the rising pattern of accumulation curve (Figure S4b), suggests that part of the diversity still needs to be sampled. It was possible to observe that the arrangements recovered on both dendrograms did not show the same pattern for the most sampled points, although points 2, 4 and 7 showed the same patterns for prokaryotes and eukaryotes (Figure 5). No spatial concordance was found between Prokaryotic and Eukaryotic microbiota communities, which was indicated also by Mantel test ($r = 0.02$, $p = 0.39$). These results demonstrate that, for a better understanding of microbiota biodiversity in freshwater ecosystem, it is necessary to analyze both metabarcoding taxonomic groups.

DISCUSSION

In the present study, we identified and classified a high number of eukaryotic and prokaryotic units dispersed in eight lakes environmentally heterogenous along the of Araguaia River, based on metabarcoding techniques. In addition, the analyses of the 16S rDNA and 18S rDNA microbiotas did not show spatial concordance. In other words, one community cannot predict the spatial arrangement of the other community in this freshwater environment. The species accumulation curve indicated that the prokaryotic diversity was well sampled while the accumulation curve for the eukaryotic microbiota was near of the asymptote. Thus, the biodiversity analysis and spatial concordance were not affected by incomplete inventories, since new samples would not increase the number of OTUs for both groups.

When assessing the biodiversity of freshwater community through metabarcoding approaches it is not always possible to identify at the species level. Nonetheless, often the identification at a higher taxonomic level is sufficient (Coissac et al. 2012) for a biodiversity and community study. In addition, the coupling of traditional and metabarcoding approaches for OTU identification, allows the evaluation of aquatic biodiversity and increases information for the management and conservation of aquatic environments. In a taxonomic study previously conducted in Araguaia lakes, 115 phytoplanktonic and 159 zooplanktonic species were found using morphological approaches (see Machado et al. 2015). Herein, although the number of sampled lakes is smaller, it was possible to recovered similar results for the most sampled higher taxa identified morphologically by Machado et al. (2015).

The taxonomic prediction of OTUs is totally dependent on the existence of comprehensive and reliable taxonomic reference data for each barcoding gene (Thomsen & Willerslev 2015, Grzebyka et al. 2017). Here, 51% of eukaryotic OTUs and 67% of prokaryote OTUs were identifiable to at least in one taxonomic category. The existence

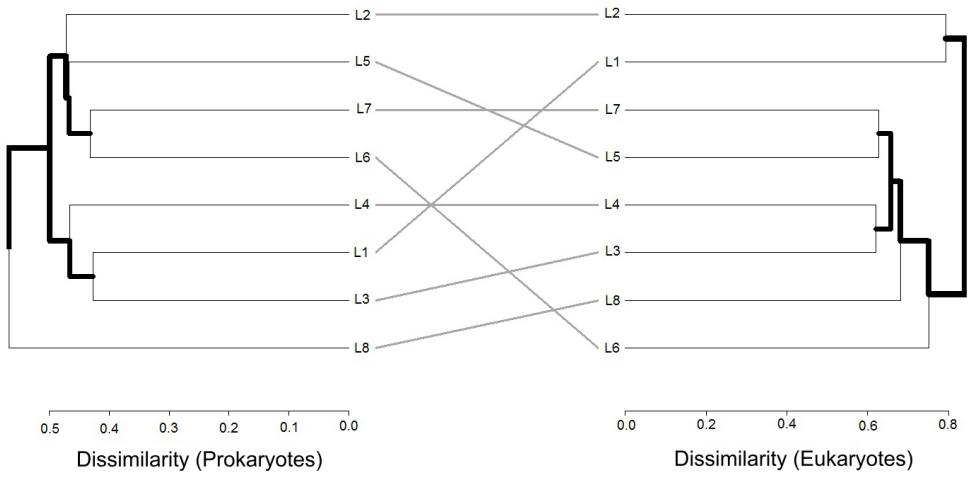

Figure 5. Dissimilarity analysis. Dissimilarity analysis for prokaryotic and eukaryotic microbiota between the floodplain lakes (L) of the Araguaia river.

of a high number of eukaryotes and prokaryotes not studied in freshwater ecosystems can explain the large proportion of OTUs detected in Araguaia river water that did not correspond closely with reference sequences in the SILVA database. This highlights the need for more inventories in this region, so that it is possible to better understand the biodiversity of these ecosystems.

The census presented here is one of the first to use metabarcoding to characterize part of the biological diversity existing from several sites of the Araguaia river (but see Tessler et al. (2017) for prokaryotes and Machado et al. (2019) and Lentendu et al. (2019) for microeukaryotes). For prokaryotic diversity, five phyla found to be present in freshwater in Brazilian floodplains by Tessler et al. (2017) were also identified here, which were: Proteobacteria, Actinobacteria, Bacteriodetes, Verrucomicrobia and Cyanobacteria. For microeukaryotes, we found 11 taxonomic groups, among the 23 observed by Machado et al. (2019) for the Araguaia River floodplain, and five phyla similar to those found by Lentendu et al. (2019) in the same region, although six taxonomic groups observed in our study are different. The presence of similar groups between studies indicates a concordance in the results and, also, that different sampling techniques (filter pore size, amount of filtered water) may represent the biodiversity existing in the region. In this context, our study adds information about planktonic communities in this underexplored region, using a metabarcoding approach.

Among prokaryotic organisms, some species of Cyanobacteria are known for their toxic potential, while many other bacteria can act as pathogens in plants, humans or other animals. In our study, we observed a low proportion of Cyanobacteria in relation to the other groups (only 12 OTUs), indicating that the issue of cyanobacterial toxicity may not be as pronounced for this region. Another factor that can contribute to the low abundance of Cyanobacteria are the high levels of turbidity found in the sampled lakes (mean = 36 NTU; ± coefficient of variation = 109%) and other lakes in the middle Araguaia river during the rainy season (e.g. Machado et al. 2015, Marcionilio et al. 2016). Furthermore, we also observed the presence of some taxa capable of causing diseases in humans such as Ricktissiales (14 OTUs; Schrallhammer et al. 2013), Moraxeleaceae (5 OTUs; Teixeira & Merquior 2014), or in plants, such as Acidovorax (1 OTU; Alves et al. 2010). However, all these have been observed at low frequencies.

The absence of concordance between prokaryotes and microeukaryotes on the same points demonstrate the importance of using different genes to detect the whole organisms and, further ahead, be used for biomonitoring a freshwater ecosystem. In this sense, they can be useful tools in the sustainable management of aquatic environments (Bradford et al. 2013, Oulas et al. 2015). Despite the separation of the lakes in terms of their environmental conditions in PCA analysis, at the sampled points there is still no strong environmental gradient. This separation was probably due to the geographic location of the lakes, which produce small gradients in the limnological variables, due to the natural variation of these parameters along the middle stretch of the river. In fact, lakes 1 to 4 are between the city of Luiz Alves and Aruanã, while points 5 to 7 are in opposite direction of the river, in the region of the Bananal Island. Thus, the absence of spatial concordance among the communities can be explained by the fact that eukaryotes and prokaryotes have distinct environmental requirements, even coexisting on a gradient with little environmental variability.

In general, our study characterized eukaryotic and prokaryotic microbiota in floodplains of the Araguaia River, adding new information for the knowledge of biodiversity in this region and complementing the information obtained through inventories based on morphological identification. We found that the sampled section is still in good condition, with the absence of potentially toxic taxa. Thus, these results contribute to the knowledge of aquatic biodiversity in the tropical region, still little studied.

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SUPPLEMENTARY MATERIAL

Figures S1, S2, S3, S4.

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JCN, LCGV, MPCT and TNS planned the work. KBM, JCN and LCGV collected the samples at the floodplains and contributed to the ecological analyses. CPT, AMA and JGF generated the genetic data. CPT, AMA and TNS wrote the first draft of the manuscript. MPCT, RL, LCGV gave support for the work and analyses.