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Cover: A Southern Rockhopper Penguin *Eudyptes chrysocome* stands on Tussock Grass on Westpoint Island. Painted in poster colors, this artwork is a reproduction of a photograph by Phillip Colla. Thanks to the photographer for the original image. © Pooja Patil.

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ARTICLE

Cataloguing biodiversity of freshwater communities in two lakes of Gadchiroli area of central India using environmental DNA analysis

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Abstract: We investigated eukaryote biodiversity in two freshwater lakes in the Aashti area of Gadchiroli in central India, using next-generation sequencing-based technology. In this preliminary study, we analyzed four water samples using metabarcoding of the 18s V6 region of mitochondrial DNA, and detected >500 operational taxonomic units (OTUs). We detected algae, dinoflagellates, rotifers, ciliates, and metazoan species and our results indicate that algae and rotifers were the most abundant groups in these lakes.

Keywords: 18S DNA barcoding, alpha diversity, beta diversity, biodiversity, environmental parameters, freshwater ecology, phytoplankton, zooplankton.

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Author contributions: MS, PC, MSP involved in the designing experiments, conduct of experiments, data analysis and manuscript writing. All authors have read and approved the final manuscript.

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INTRODUCTION

Phototrophic algae, heterotrophic protists, rotifers, crustaceans, dinoflagellates, and diatoms usually dominate the freshwater microscopic eukaryotic communities (Manabe et al. 1994; Nishikawa et al. 2010), and play a crucial role in governing the biogeochemical cycles in the lotic and lentic waterbodies (Allan 1976; Gannon & Stemberg 1978). Phytoplankton and zooplankton play essential roles in C and N cycles, and enhance the stability of aquatic ecosystems (Steinberg et al. 2008). Zooplankton directly feeds on phytoplankton and thus contributes to the inhibition of the eutrophic conditions in lakes (Cottenie et al. 2003; Kohout & Fott 2006; Schou et al. 2009). Similarly, many zooplankton are sensitive to anthropogenic stressors, and thus can serve as useful biological indicators of environmental stressors (Beaugrand et al. 2002; Grosjean et al. 2004; Blanco-Bercial & Bucklin 2016). Marine, wetland, and freshwater ecosystems are facing various threats to their stability, including toxicant pollution, nutrient influx, land use, and climate change. It is known that these human activities change the biogeochemical cycles, which in turn change the types of species that live in freshwater ecosystems, and how those ecosystems work (Baldwin et al. 2014; Drake 2014). Anthropogenic activities significantly altered the population dynamics and biodiversity of aquatic habitats (Sala et al. 2000). Conservation efforts are hampered by a lack of detailed information on biodiversity and the rates of species extinction in freshwater ecosystems (Ricciardi & Rasmussen 1999; Pimm et al. 2014). Therefore, protecting the aquatic ecosystems and their biodiversity is of prime importance, and concentrated efforts are required to conserve these precious ecosystems. In this context, documenting the true biodiversity in various ecosystems is essential.

Several studies on cataloguing phytoplankton and zooplankton diversity are available in the literature (Banse 1995; Nogueira 2001; Branco et al. 2002; Neves et al. 2003; Whitman et al 2004; Mageed 2007; Frutos et al. 2009; Suresh et al. 2011; Vanderploeg et al. 2012; Paturej et al. 2017; Gao et al. 2019; Li et al. 2019). Plankton diversity of different aquatic ecosystems has been identified using DNA barcoding (Amaral-Zettler et al. 2009; Bucklin et al. 2019; Machida et al. 2009; Tang et al. 2012; Hadziavdic et al. 2014; Djurhuus et al. 2018; Wangensteen et al. 2018; Berry et al. 2019). Traditional taxonomic methods have been used by Indian researchers to record the different aquatic communities in a number of freshwater habitats (Madhupratap et al. 1981; Mishra et al. 1993; Jha & Barat 2003; Kiran et al. 2007; Kumar et al. 2011; Harney et al. 2013; Smitha et al. 2013; Jyotibabu et al. 2018; Bhattacharya et al. 2015; Manickam et al. 2018). The limitations of traditional taxonomic methods in identifying microscopic forms have hindered the complete elucidation of the true plankton diversity in these freshwater lakes and ponds. Recently, few studies employed DNA barcoding to explore plankton biodiversity (Nair et al. 2015; Govender et al. 2022). Few studies have used metagenomics to identify diversity in freshwater lakes in India. These observations suggest a need for comprehensive studies to identify the biodiversity in freshwater ecosystems of central India. In the current study we used environmental DNA barcoding to catalogue eukaryote diversity in two freshwater lakes from the Gadchiroli area of central India.

MATERIALS AND METHODS

Sampling sites

Two lakes, Chandankhedi Lake 1 (ASL1, 19.709° N & 79.826° E) and Chandankhedi Lake 2 (ASL2, 19.726° N & 79.833° E), are situated near Chandankhedi Village, Ashti area, Gadchiroli District, Maharashtra State of India (Figure 1). The ASL1 and ASL2 are not included in any area that is reserved for biodiversity conservation or privately owned, so no specific permissions were required to conduct the sample collection. The current study did not collect or include any species listed as endangered or protected in species lists. Since the schedule species list of animals does not include the organisms in the plankton sample, no ethical committee approval was required. We followed the collection procedures as outlined in the literature (Harris et al. 2000).

Water samples

We collected a one-liter water sample from three different depths near the lake's periphery (littoral zone) and inside the lake (limnetic zone) in sterile collection bottles and processed it within a day. The three samples collected from the periphery (littoral zone) of each lake were combined and labeled as ASL1P, and ASL2P. Similarly three samples from the interior (limnetic zone) of each lake were combined and labeled as ASL1I, and ASL2I. A total of four samples ASL1P, ASL2P, ASL1I, and ASL2I were processed for metagenomics analysis. Chemical parameters estimated for water samples included hydrogen ion concentration (pH) and total dissolved solids (TDS), recorded using portable meters (Amstat, USA). Other chemical parameters were estimated in

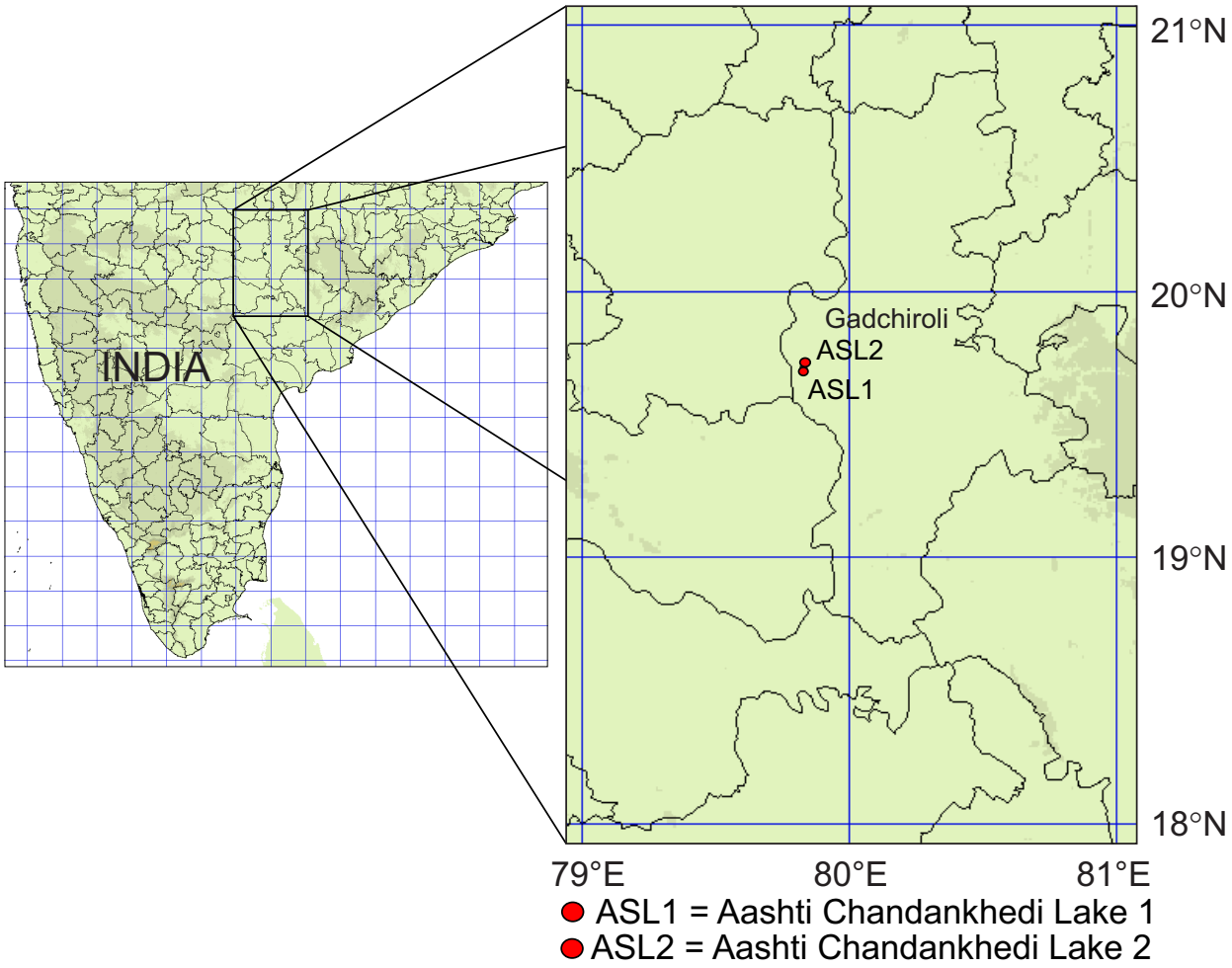

Figure 1. Collection sites: Chandankhedi, Aashti Lake 1 (ASL1), and Chandankhedi, Aashti Lake 2 (ASL2).

the laboratory using standard protocols (APHA 2008). Winkler's method was used to measure dissolved oxygen (DO), and titrimetric methods to measure free CO_2 and total hardness. We estimated total alkalinity using titrimetric methods by combining two values: free CO_2 (carbonate alkalinity) and bicarbonate alkalinity, measured with phenolphthalein, and methyl orange indicators, respectively, and titrating the water sample against N/50 sulphuric acid.

DNA extraction

DNA extraction from the collected samples: ASL1 P (littoral zone) and ASL1 I (limnetic zone) from Chandankhedi Lake 1, and ASL2P (littoral zone) and ASL2I (limnetic zone) from Chandankhedi Lake 2 was performed using the DNA Easy Power Water DNA Isolation Kit (Qiagen, USA). DNA isolation was carried out according to the manufacturers' protocol. The genomic DNA was checked on a 1% agarose gel for the presence of a single intact band. Further, 1 µL of each sample was loaded in a microvolume spectrophotometer for determining the A260/280 ratio (Denovix, USA). The DNA was quantified using a QuantiFluor® ONE dsDNA System (Promega, USA).

Amplification of the 18S rRNA gene and subsequent Illumina sequencing

The amplicon sequencing protocol targeting the V4 region of the 18S gene was used to prepare the sequencing libraries for metagenomics analysis. DNA amplicon libraries were generated according to the guidelines provided by Illumina (http://www.illumina.com). The forward and reverse primers, possessing adapter amplicon lengths compliant with Illumina standards, were produced, and utilized for amplification. The PCR reactions were conducted under these conditions: initial denaturation at 95°C for 15 minutes, followed by 35 cycles consisting of denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for one minute.

The amplification concluded with a final extension phase at 72°C for 10 minutes. The PCR products were purified with a column-based purification kit (Promega, USA), analyzed via gel electrophoresis to confirm size, and quality, and quantified using a QuantiFluor® ONE dsDNA System (Promega, USA). Indexing PCR, ampure bead purification, equimolar pooling, and sequencing on the Illumina 250 PE platform were conducted at the FirstBase DNA Sequencing Service in Malaysia. Libraries were sequenced utilizing the paired-end Illumina 250 PE platform to provide 250 bp paired-end raw reads. The paired-end reads of each sample were cleaned by removing the barcodes and primer sequences, and were merged using FLASH (V1.2.7) (Lozupone et al. 2007). We performed quality cleanup on the raw tags using specific filtering parameters, resulting in high-quality clean tags (Avershina et al. 2013, Qiime (V1.7.0); Magali et al. 2013). The chimeric sequences were eliminated to get high-quality tags for bioinformatics and taxonomic research (Edger et al. 2011).

OTU cluster and taxonomic annotation

Sequence analysis was carried out using all the effective tags employing the Uparse software (Uparse v7.0.1090, Magoč et al. 2011). Sequences having more than 97% similarity were considered as the same OTUs. A representative sequence for each OTU was checked for further annotation. Sequence analysis was carried out using the Qiime RDP method (Version 1.7.0, http://qiime.org/scripts/assign_taxonomy.html; Bokulich et al. 2013). The Silva database (http://www.arb-silva.de; Caporaso et al. 2010) was used for species annotation (Threshold: 0.6~1). Sequences were aligned using MUSCLE (Version 3.8.31, http://www.drive5.com/muscle; Edgar 2013) to obtain phylogenetic relationships. We selected the top 100 genera to understand the phylogenetic relationships. OTU abundance was normalized using a standard of sequence number equivalent to the sample with the least sequences. We performed subsequent analyses of alpha diversity and beta diversity using the normalized data.

Statistical analysis

Alpha diversity indices, observed species, Shannon, ACE, Chao1, Simpson, and good coverage, were calculated using QIIME (Version 1.7.0). We calculated beta diversity on both weighted and unweighted UniFrac using the QIIME software (Version 1.7.0). A square matrix of "dissimilarity" or "distance" was calculated and used for non-metric multidimensional scaling (NMDS) analysis, and principal coordinate analysis (PCoA). AMOVA was estimated by mothur using the amova function. Canonical correspondence analysis (CCA) was performed to understand whether there was any relationship between OTU and the chemical parameters. A scatter plot was graphed to understand the contribution of each CCA axis. The significance of canonical correlations was tested at two levels using 999 permutations (Legendre & Legendre 1998). The significance of the trace value was estimated to test the overall null hypothesis that there is no correlation between the environmental parameters and the species occurrence, and (2) the significance of individual canonical eigenvalues was tested with the same null hypothesis but against the alternate hypothesis that a given eigenvalue explains more of the variation of species occurrence than matrices with permuted rows would.

RESULTS

Assignment of Molecular Operational Taxonomic Units (OTUs)

We generated and sequenced amplicons of the 18S small subunit rRNA gene for each sample. A total of 1,105,618 DNA sequences were generated. After quality control and removal of chimeras, 994,568 good-quality sequences remained (Table 1). The average read length for the sequencing reads was 311 bp. Using a 97% similarity cut-off, the clean read tags were clustered into a total of 642 OTUs. We recorded a total of 568 OTUs in Chandankhedi Lake 1 (ASL1) and 437 OTUs in Chandankhedi Lake 2 (ASL2) (Figure 2 A, Supplementary Information S1). All four samples shared 189 OTUs, while the ASL1 sample had the highest number of unique OTUs (Figure 2B). The ASL1 sample displayed the highest number of unique OTUs (Figure 2B). Of the observed OTUs from two lakes, only 163 were identified at the species level. Arthropoda was the most abundant group, and Rotifera was the second most abundant taxon (Figure 3A). The least diverse taxonomic group was Euglenozoa. Maxillopoda, Monogononta, Chrysophyceae, and Intramacronucleata were the most dominant classes, whereas Calanoida, Cyclopoida, Flosculariaceae, and Ploimida were the most abundant orders in ASL1, and ASL2 (Figure 3B). Calanoida, Cyclopoida, Flosculariacea, and Ploimida were the most dominant families, whereas *Calanoida, Cyclopoida, Flosculariacea,* and *Ploimida were the* most abundant genera (Figure 3C). *Mesocyclops dissimilis, Ptygura libera, Vallisneria natans, Filinia longiseta, Limnias ceratophylli, Nymphoides peltata, Sphaerastrum fockii, and Collotheca campanulata were*

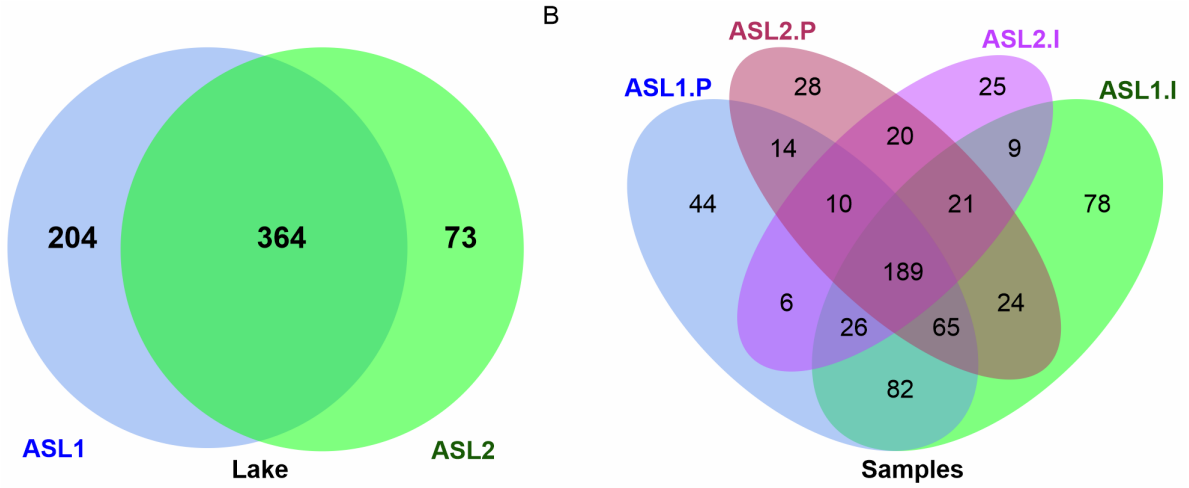

Figure 2. Biodiversity of Chandankhedi, Aashti lakes: A—Venn diagrams illustrating the number of common and unique OTU between Chandankhedi, Aashti Lake 1 (ASL1), and Chandankhedi, Aashti Lake 2 (ASL2) | B—Venn diagrams illustrating the number of common and unique OTU between four samples (ASL1.I, ASL1.P, ASL2.I, and ASL2.P).

Table 1. QC statistics of ASL1 and ASL2 samples.

Sample name	Raw PE(#)	Raw Tags(#)	Clean Tags(#)	Effective Tags(#)	Taxon Tag	Average length (nt)	OUT number	Species	Effective %
ASL1.I	284,836	275,043	273,629	262,811	261716	311	513	494	92.27
ASL1.P	271,293	263,039	261,914	245,710	244777	311	460	436	90.57
ASL2.I	272,095	262,053	260,807	235,995	234697	311	339	306	86.73
ASL2.P	277,394	266,638	265,350	250,052	249131	311	400	371	90.14

the most common species.

Alpha and beta diversity

Alpha and beta diversity analyses of ASL1 and ASL2 sequence reads revealed rich taxonomic diversity and dominance of a few species (Figure. 4, Supplementary Information S2). Shannon's index ranges from 1–1.5, indicating high species richness in the samples collected from these lakes (Figure 4A). Interestingly, samples from ASL1.P (D = 0.296), ASL1.I (D = 0.32), ASL2.P (D = 0.209), and ASL2.I (D = 0.193) showed higher dominance among fewer groups (Figure 4B). The ACE analysis showed that the lake samples had a lot of different species (Figure 4C), and the Chao-1 analysis predicted that these samples would have between 337 and 511 different species (Figure 4D). Alpha diversity indices such as the Shannon index, evenness, and Margalef index were not significantly different between the ASL1 and SL2 lake samples (Mann-Whitney *U* test *P* >0.05 for each comparison). Interestingly, the Simpson index showed a significant difference between ASL1 and ASL2 (Mann-Whitney *U* test, *P* <0.05). Beta diversity analysis indicated that the composition of species in these two lakes is significantly different (Figure 4E; nMDS Stress <0.001). A species accumulation curve showed the presence of 642 OTUs in these lake samples (Figure 4F). The analysis of molecular variance (AMOVA) revealed no significant difference in molecular variance between the samples collected from ASL1 and ASL2 lakes (Fs = 6.72682, p = 0.342).

Correlation between species composition and biochemical characteristics of lakes

The composition and biodiversity of eukaryotes were significantly different among the two lakes (Figure 2). NMDS analysis indicated that biological diversity in these two lakes clearly discriminated from each other (Figure 4E, Trace p <0.01). Proportions of Rotifera, Ochrophyta, Ciliophora, Cryptomycota, Diatomea, Chlorophyta, Phragmoplastophyta, and Peronosporomycetes differed significantly among water bodies. Canonical correspondence analysis suggested that there was a strong correlation between chemical parameters and species occurrence (Figure 5, trace = 0.00087, P = 0.039). The first two axes, which together explained 93.8% of the total inertia, were significant,

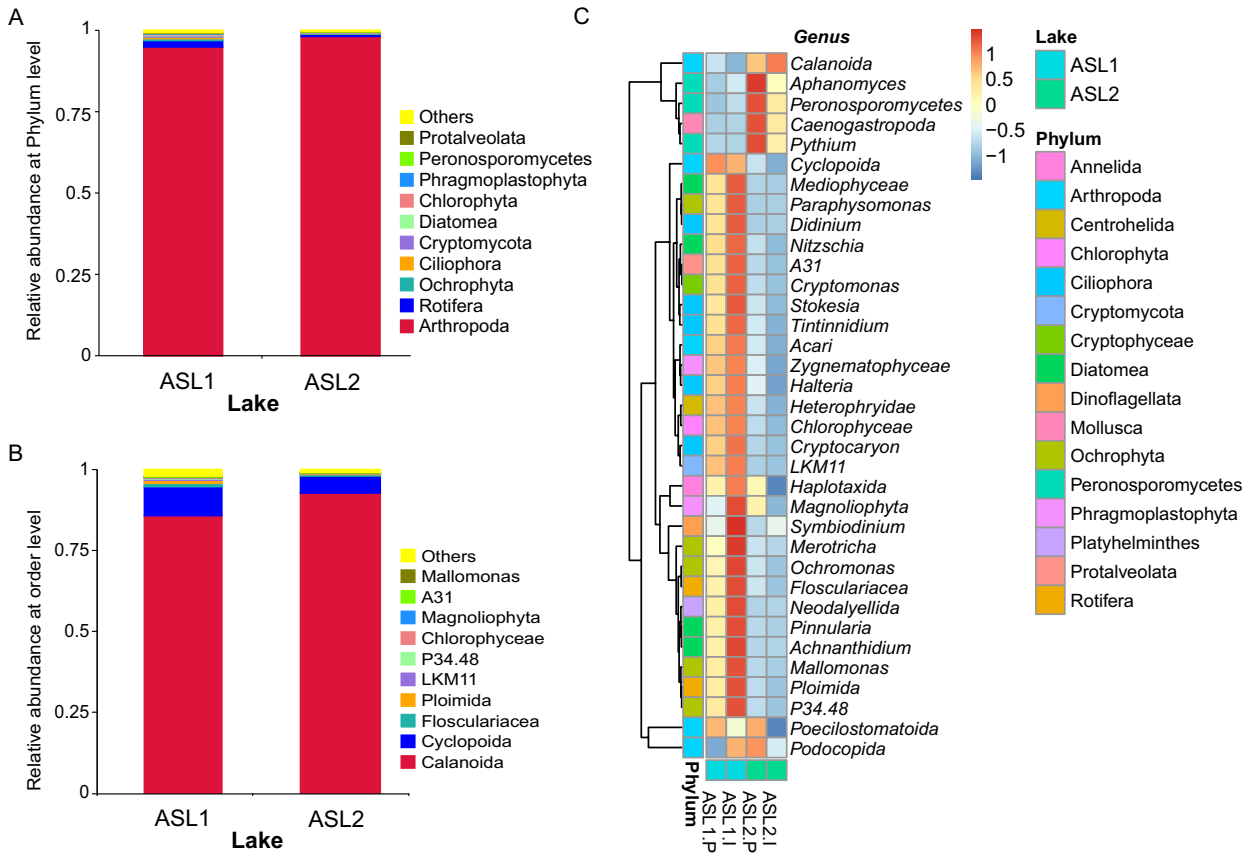

Figure 3. Species composition in Chandankhedi, Aashti Lake 1 (ASL1), and Chandankhedi, Aashti Lake 2 (ASL2): A—Relative abundance of OUT at phylum level | B—Relative abundance of OUT at order level | C—Taxonomic abundance cluster heatmap at genus level: According to abundance information of top 35 genus of all samples, the heatmap was drawn. Sample name on the X-axis and the Y-axis represents the genus. The absolute value of 'z' represents the distance between the raw score and the mean of the standard deviation. 'Z' is negative when the raw score is below the mean, and vice versa.

and depicted the relationship between chemical parameters, and species occurrence. Most species were clustered around the origin of both axes, indicating that they had no particular preference for chemical parameters. Interestingly, only a few species showed a correlation with the chemical parameters of water. For instance, *Bryometopus atypicus, Chloromonas oogama, Malassezia globosa,* and *Cyanophora paradoxa* had preferences for relatively higher values of TDS. *Cloeon durani, Chironomus tentans, Dinobryon sp.,* and *Pinnularia sp.* showed preference for relatively higher values of total hardness, chloride, and dissolved CO2. *Pseudorhizidium endosporangiatum, Trochilia petrani, Furgasonia blochmanni,* and *Pseudocharaciopsis ovalis* showed preference for higher values of dissolved oxygen, and *Ochromonas sphaerocystis, Gieysztoria sp., Linostomella sp.,* and *Chlamydopodium starrii* showed preference for higher values of alkalinity, and salinity.

The evolutionary tree of the top 100 genera

Of the observed OTUs from two lakes, 169 OUT could be identified at genera level. Out of 169 identified genera, the top 100 were used for phylogenetic analysis (Figure 6; Supplementary Information S1). Phylogenetic analysis revealed that more than 90% of OUT reads accounted for five phyla (Calanoida, Cyclopoida, Ploimida, Flosculariacea, Philodinia), suggesting the dominance of a few phyla in ASL1, and ASL2 lakes.

DISCUSSION

Aquatic fauna of freshwater lakes plays a fundamental role in the food web and provides important information about the state of the water body (Manabe et al. 1994; Nishikawa et al. 2010). Several studies have looked at the variety of phytoplankton and zooplankton in freshwater, estuarine, and marine water bodies around the world (Banse 1995; Nogueira 2001; Branco et al.

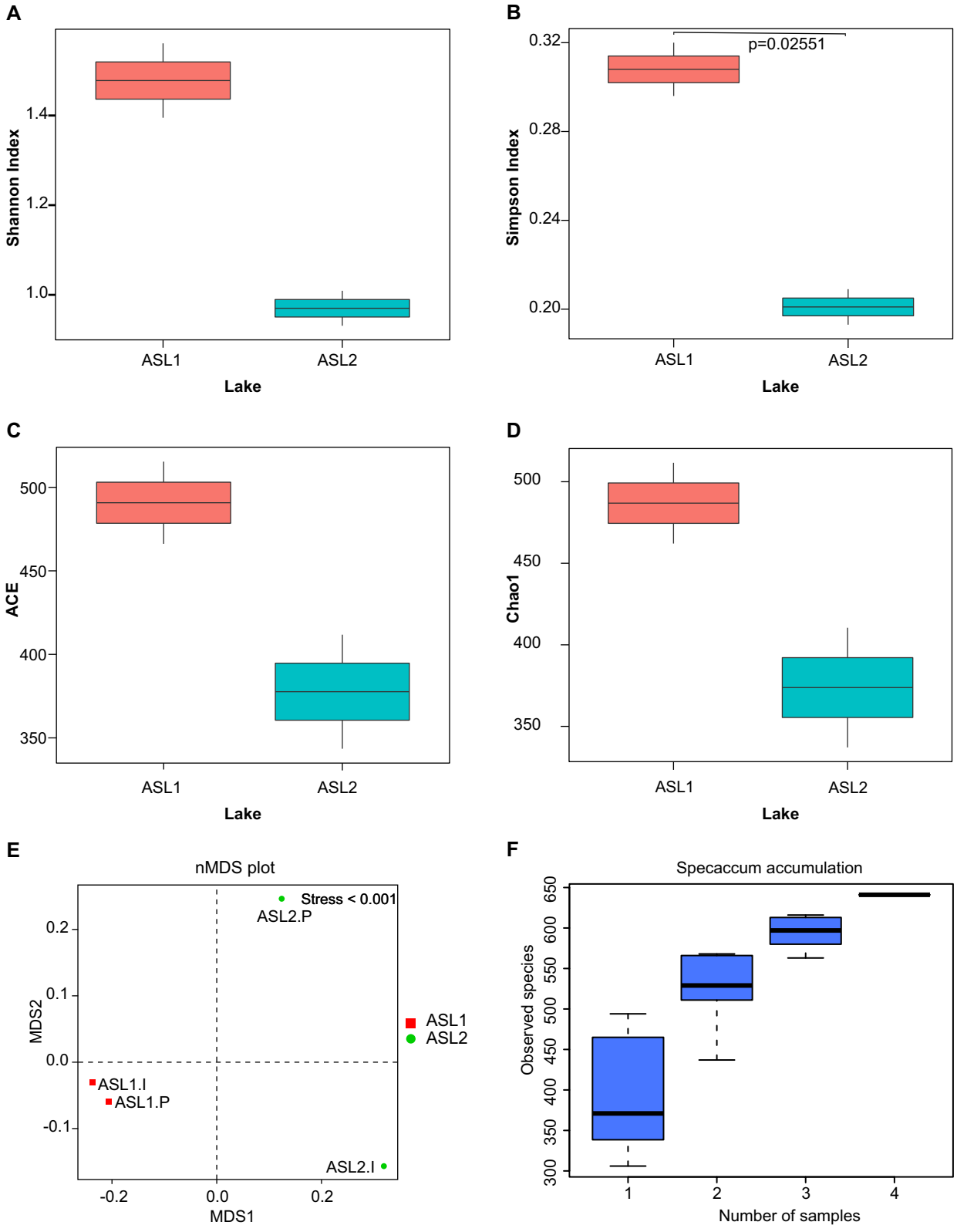

Figure 4. Alpha, beta, and gamma diversity indices: Alpha diversity box plots | A—Shanon index | B—Simpson Index | C—ACE | D—Chao1, Beta diversity plot | E—n MDS plot and gamma diversity plot | F—Species accumulation.

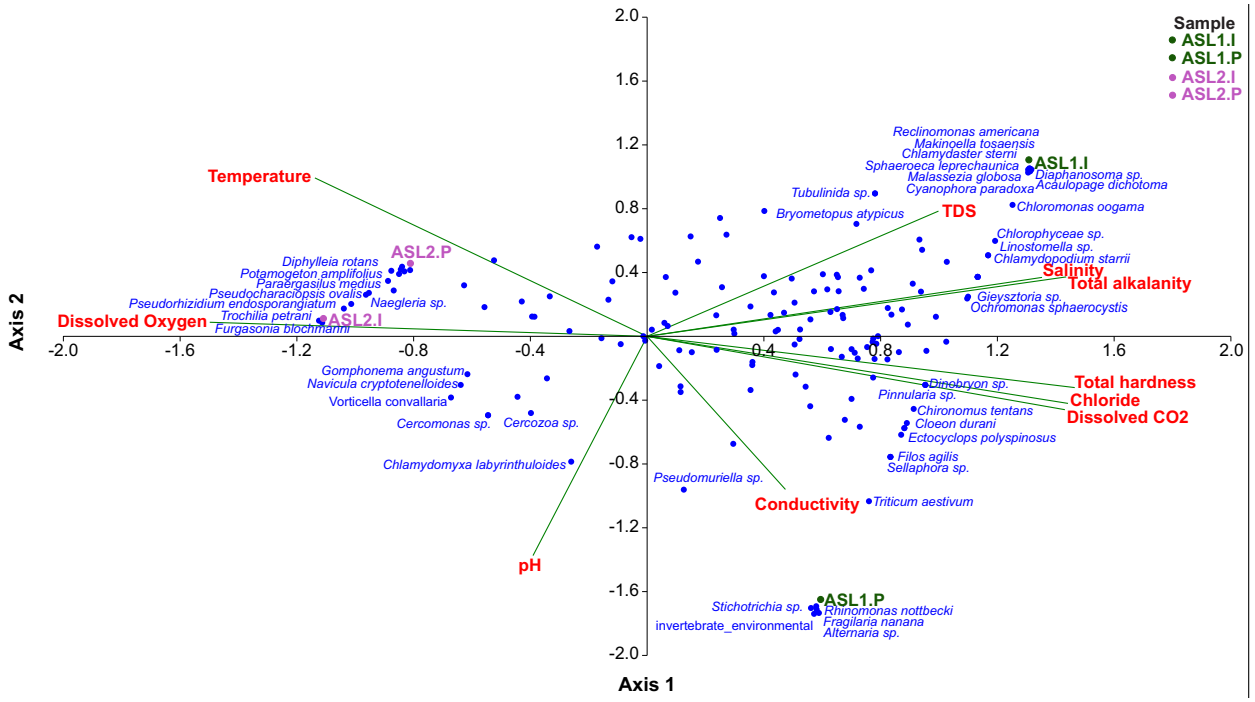

Figure 5. Canonical correspondence analysis (CCA) ordination plot for species composition, samples, and environmental variables.

2002; Neves et al. 2003; Whiteman et al. 2004; Mageed 2007; Frutos et al. 2009; Suresh et al. 2011; Vanderploeg et al. 2012; Paturej et al. 2017; Gao et al. 2019; Li et al. 2019). Several studies in India have catalogued the biodiversity of phytoplankton and zooplankton in rivers, estuaries, and marine habitats (Madhupratap et al. 1981; Mishra et al. 1993; Jha & Barat 2003; Kiran et al. 2007; Kumar et al. 2011; Harney et al. 2013; Smitha et al. 2013; Jyothibabu et al. 2015; Manickam et al. 2018; Bhattacharya et al. 2015). Taxonomic studies of these bodies of water showed that they were home to protozoa, rotifers, copepods, cladocera, ciliophora, and meroplanktons. Similarly, genetic analysis studies also documented the presence of several zooplankton and phytoplankton species in rivers and lakes of India (Nair et al. 2015; Govender et al. 2022).

The main goal of this study was to obtain taxonomic and genetic data for eukaryotes in two freshwater lakes in the Aashti area of Gadchiroli, Maharashtra. The metagenomic analysis of the lakes suggested the presence of a rich eukaryotic community structure. The universality of 18S primers and sample collection methods played a crucial role in documenting the true diversity of the aquatic forms present in the two lakes, ASL1 and ASL2. Rotifera, Cladocera, and Maxillopoda, along with other aquatic organisms, including aquatic Phragmoplastophyta, Platyhelminthes, Ochrophyta, Holozoa, Gastrotricha, Diatoms, Protista, Nematoda, Ciliophora, Diatomea, and Chlorophyta, were predominant in the sampling sites. *Eudiaptomus environmental, Mesocyclops dissimilis, Arthropoda environmental, Neoergasilus japonicus, Microcyclops varicans,* and *Unionicola foili* comprised over 90% of the total numbers of OUT (Figure 6). Rofifers, *Ptygura libera, Filinia longiseta, Limnias ceratophylli,* and *Collotheca campanulata* were abundant in these two lakes. *Vallisneria natans, Nymphoides peltata*, and *Chlamydomonas reinhardtii* dominated the plant species. Diatoms such as *Achnanthidium saprophilum* and *Urosolenia eriensis* were present in good numbers in these two lakes (Figure 6). Although DNA metabarcoding identified more than 600 OTUs in the current study, only 163 OTUs could be identified at the species level. Chao-1 analysis suggested that more than 600 species might be present in the study area. The results obtained in the current study suggest that the ASL1 and ASL2 lakes have high species diversity with a complex community structure (supplementary information, Table S1 and Figure 2), and in-depth taxonomic analysis is required to uncover the true diversity in these two lakes.

Maxillopoda has been considered a bioindicator of environmental fluctuation and ecosystem dynamics (Campos et al. 2017; Jyothibabu et al. 2018). On the other hand, Cyclopoida are capable of surviving in different habitats and maintaining their population size in hostile conditions as well (Paffenhoffer 1993). In these two lakes,

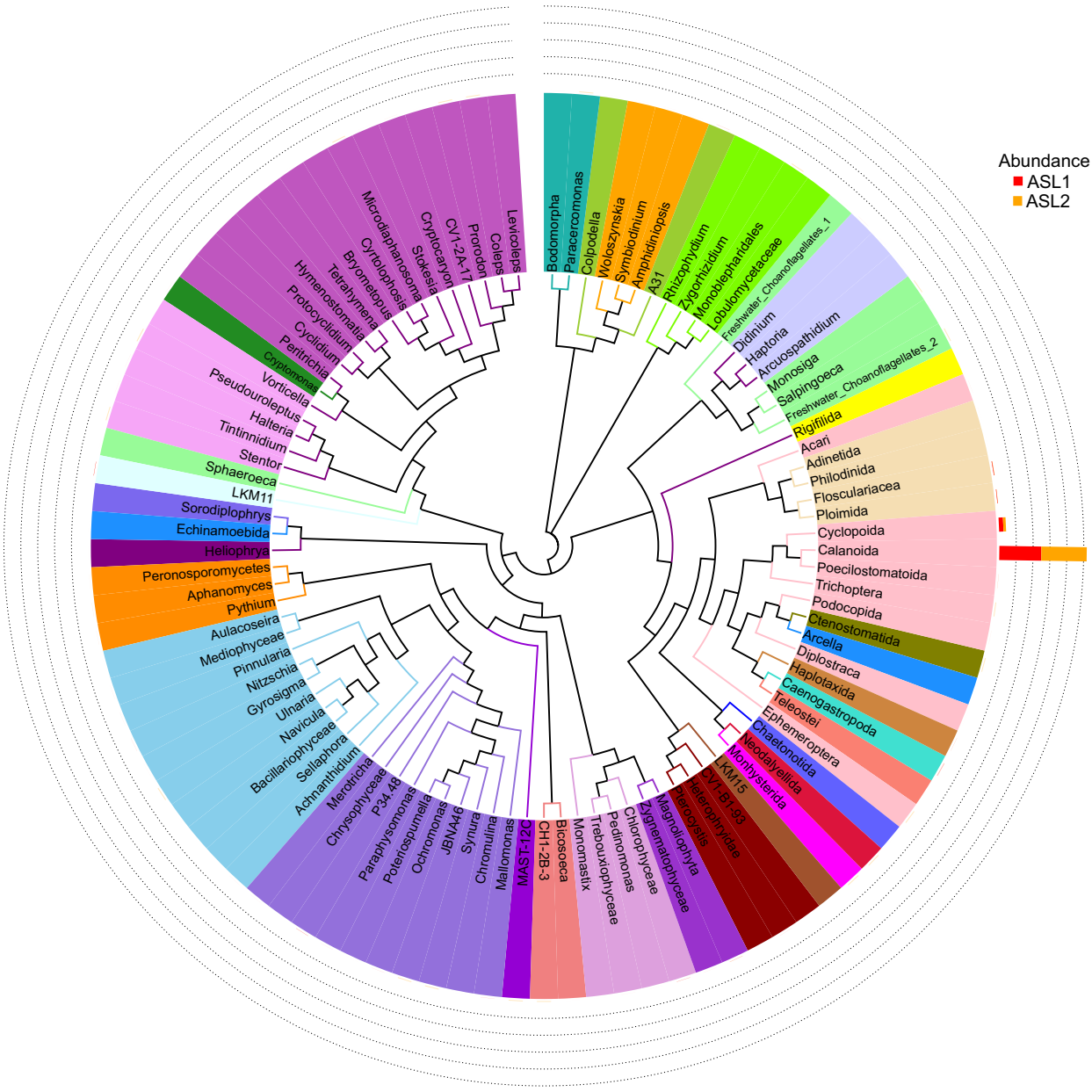

Figure 6. The phylogenetic relationship of genus: the top 100 genera were selected and the evolutionary tree was drawn using the aligned representative sequences. Different colours of the branches represent different phyla. Relative abundance of each genus in each group was displayed outside the circle and different colours represent different groups.

ASL1 and ASL2, Maxillopoda, Calanoida, and Cyclopoida were abundantly present. These observations suggest that these two lakes are experiencing fewer threats from anthropogenic activities. Although the plankton fauna has been recorded from a wide range of environmental conditions, environmental factors such as pH, dissolved oxygen, salinity, and temperature play an important role in determining the accumulation of species (Ahmad et al. 2012). Few species exhibit a profound response to a given factor, while others do not demonstrate any significant response (Figure 5). The results obtained in the current study indicated that environmental variables, dissolved CO_2, total hardness, chloride concentration, TDS, and oxygen concentration have a significant role in determining the species composition.

It has been well documented that temperature plays a crucial role in determining the diversity and abundance of plankton communities. The results obtained in the current study suggest that temperature might not be influencing the species diversity in these two lakes, ASL1

and ASL2 (Figure 5). *Bryometopus atypicus, Chloromonas oogama, Malassezia globosa,* and *Cyanophora paradoxa* showed preference for relatively higher values of TDS. On the other hand, *Cloeon durani, Chironomus tentans, Dinobryon sp,.* and *Pinnularia sp.* showed preference for higher values of total hardness, chloride, and dissolved CO_2. *Pseudorhizidium endosporangiatum, Trochilia petrani, Furgasonia blochmanni,* and *Pseudocharaciopsis ovalis* prefer higher values of dissolved oxygen for survival in lake environments. On the other hand, *Ochromonas sphaerocystis, Gieysztoria sp., Linostomella sp.,* and *Chlamydopodium starrii* showed affinity for higher values of alkalinity, and salinity. The observations corroborate the results obtained in the earlier studies.

The use of the Illumina platform enabled us to detect several operational taxonomic units (OTUs) of eukaryotes using environmental DNA, even though they are available in low abundance in samples. The outcome of this study revealed that we have significantly underestimated plankton diversity in the past due to too much reliance on traditional microscopy-based methods. The results obtained in this study are preliminary in nature and require further investigation.

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