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**Short Communication**

# Metabarcoding of Freshwater Green Microalgae from Two Urban Streams in the Philippines

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## ABSTRACT
Green microalgae (Chlorophyta) are found worldwide in various environments. Currently, there are few studies on Philippine freshwater green microalgae and traditional identification methods using morphology and life cycle characters are time consuming and may be unreliable. This study used eDNA and Illumina sequencing to sample algal communities in two urban streams situated in a local university campus and found 39 chlorophyte genera. Three markers were used and each marker detected taxa not found by the other markers, suggesting that a multimarker approach might be best for future metabarcoding studies and that other gene markers should be tested for utility.



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Green microalgae (Chlorophyta) are ubiquitous dwellers of terrestrial and aquatic ecosystems, found in both freshwater and marine ecosystems, and are commonly used for water quality assessments. They are a diverse lineage with an estimated 5000 undescribed species (Guiry 2012). The study and identification of algae have traditionally relied on morphology and culture characteristics, such as cell shape, life cycle, and other physical characteristics (Zou et al. 2016). However, these characters often exhibit plasticity and are not always reliable, especially with regard to cryptic taxa (Cianciola et al. 2010).

Studies of freshwater green microalgae in other countries have recently started using metabarcoding as a tool for surveying biodiversity (Jacobs-Palmer et al. 2021; Bukin et al. 2022). Similar to traditional DNA barcoding (Zou et al. 2016), metabarcoding utilises short gene sequences for molecular identification and can identify multiple species from a single sample (Kezlya et al. 2023). Metabarcoding, using multiple DNA markers for rapid biodiversity assessments, has proven effective and multiple metabarcoding projects for green algae have been conducted internationally (Groendahl et al. 2017; Jacobs-Palmer et al. 2021; Bukin et al. 2022).

There have been several limnological surveys in the Philippines that also identified freshwater green microalgae, such as Papa and Mamaril's (2011) survey of Taal Lake and a microalgal survey of the Rio Grande de Cagayan (Baleta et al. 2016). However, these studies have mostly relied on morphology for identification, and to date, no metabarcoding studies have been published for Philippine freshwater green microalgae.

This study presents the first application of metabarcoding to Philippine freshwater green microalgae sampled from urban streams. Results from traditional, culture-based identification are also briefly discussed.

Water sampling from urban streams in the University of the Philippines Institute of Biology Arboretum (AR; 14°39.00′N, 121°4.14′E) and along the University Avenue (UA; 14°39.24′N, 121°3.6′E) was conducted in February 2022 and March 2023, respectively. Samples were collected using one-litre Pyrex glass bottles that had been bleached, and washed with distilled water, and then autoclaved for sterility. Two replicates of three litres each were collected at each site. Surface and subsurface (< 5 cm depth) water were collected; however, both streams were shallow (< 0.5-metre depth at the sampling site) and gently flowing, so some sediment might have mixed in.

Water samples were vacuum-filtered using 20 µm nylon sieves (Sigma Aldrich, USA) to capture environmental DNA. DNA extraction from the sieves was performed by mashing the sieves using glass beads in 700 µL of lysis buffer (Zymo Research, USA) and a bead basher (Disruptor Genie, Scientific Industries, USA) at 328 × g (3,000 rpm) for 10 minutes. The resulting mixture was centrifuged at 20,000 × g for 10 minutes and the supernatant was collected. DNA purification was performed using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) following the manufacturer's protocols, with only the bead bashing step modified as previously indicated. Samples were then sent to Macrogen Inc. (Seoul, South Korea) for library preparation and Illumina MiSeq 250 base-pair (bp) paired-end sequencing. Primers with proven utility for algal metabarcoding, such as the V4 and V9 regions of the 18S nuclear gene and the 23S plastid gene (Bukin et al. 2022; Kezlya et al. 2023) were used for DNA amplification.

For both sampling locations, the V4 and V9 regions (Table S1) of the 18S gene were selected as standard primers due to their ease of amplification and relatively small (less than 600 bp for V4, and less than 200 bp for V9) product size. For the AR samples, the 23S markers were also tested for their utility in metabarcoding (Table S1). The 23S marker could not be tested at both sites due to funding constraints.

For downstream analysis, reads from both replicates at each sampling

site were pooled. Raw Illumina reads were filtered and trimmed using *Cutadapt* v4.4 (Martin 2011) to remove short reads, primers, and Illumina adapters. Filtered reads were analysed using the dada2 v1.16 pipeline (Callahan et al. 2016) in R v4.3. Merged reads for the V4 and V9 markers were aligned to the SILVA database v132 (Callahan 2018). A custom database was made for 23S by downloading chlorophyte sequences from GenBank. Sequences were formatted according to the SILVA database for use in the dada2 pipeline. Bootstrap support for database matching was set to the dada2 package's default 50 % threshold.

For GenBank, the search terms for 23S were "((((Chlorophyta) OR Chlorophyta [Organism]) AND 23S[All Fields]) AND 100:10000[SLen] NOT "shotgun" [All Fields]". Also, 23S sequences from two chloroplast genomes (MF197536 and NC_045059) were added to the final database.

The resulting classifications for V4, V9, and 23S were filtered to retain only Chlorophyta sequences using the phyloseq R package v1.41 (McMurdie & Holmes 2013). Graphs illustrating which green algal genera were identified by each markerand diversity index computations were generated in Microsoft Excel. Pooled Illumina reads were uploaded to the Sequence Read Archive of GenBank (Table S2).

Water samples from both locations were also plated on Bold's Basal Medium with 1 % kanamycin and 1 % nystatin. Cultured algae were viewed under light microscopy for morphological examination, and identity was further confirmed using the tufA marker (Table S1).

Reads from the V4 primers, the majority of which range from 477 to 518 bp, resulted in 1659 Amplicon Sequence Variants (ASVs). Of these, 216 were classified as Phylum Chlorophyta sequences when aligned to the SILVA database (v132), and 102 ASVs were further classified downto genus level, resulting in 26 genera (Figure 1A) among 19 families. The remaining 114 chlorophyte sequences were not matched to genus level and were discarded for further analysis.

The V9 primers yielded 2967 ASVs. A total of 82 ASVs were classified as Phylum Chlorophyta while 18 ASVs were classified down to genus level, resulting in 15 genera (Figure 1B) distributed among 14 families.

Processing of reads from the 23S primers resulted in 1185 ASVs. 45 of these ASVs were non-chlorophyte eukaryotes, while an additional ASV was classified as a Prasinodermophyte. Of the remaining 1139 chlorophyte ASVs, 102 were classified down to genus level by the dada2 classifier, with nine genera distributed among eight families from the AR samples (Figure 1C). The 23S marker was not used for the University Avenue stream samples; therefore, there are no results from that site.

A total of 36 chlorophyte genera were detected by V4, V9, and 23S (Table 1). Some overlaps in taxon detection exist among each marker. *Oedogonium* and *Chlamydomonas* were detected by all three markers, while 10 other taxa were detected by at least two markers. The remaining 24 are unique to a specific marker, with 15 of those detected only by V4, five by 23S, and four by V9.

Samples from the Arboretum were somewhat more diverse as compared to the University Avenue based on Shannon H-values (Table 2) for both V4 and V9 primers. There is however a concern that the Shannon value for V4 is due to the overamplification of *Spermatozopsis* sequences, with 65 of the 102 chlorophyte ASVs matched to this genus. This is possibly a result of PCR bias, given that no other marker showed the same over abundance for this species in this study. The 65 ASVs for *Spermatozopsis* were matched to over 59,000 reads, with the next most abundant genus, *Micractinium*, comprised of only two ASVs with 449 matches (Figure 1A). Removing *Spermatozopsis* counts results in a nearly equal Shannon index value for AR and UA for the

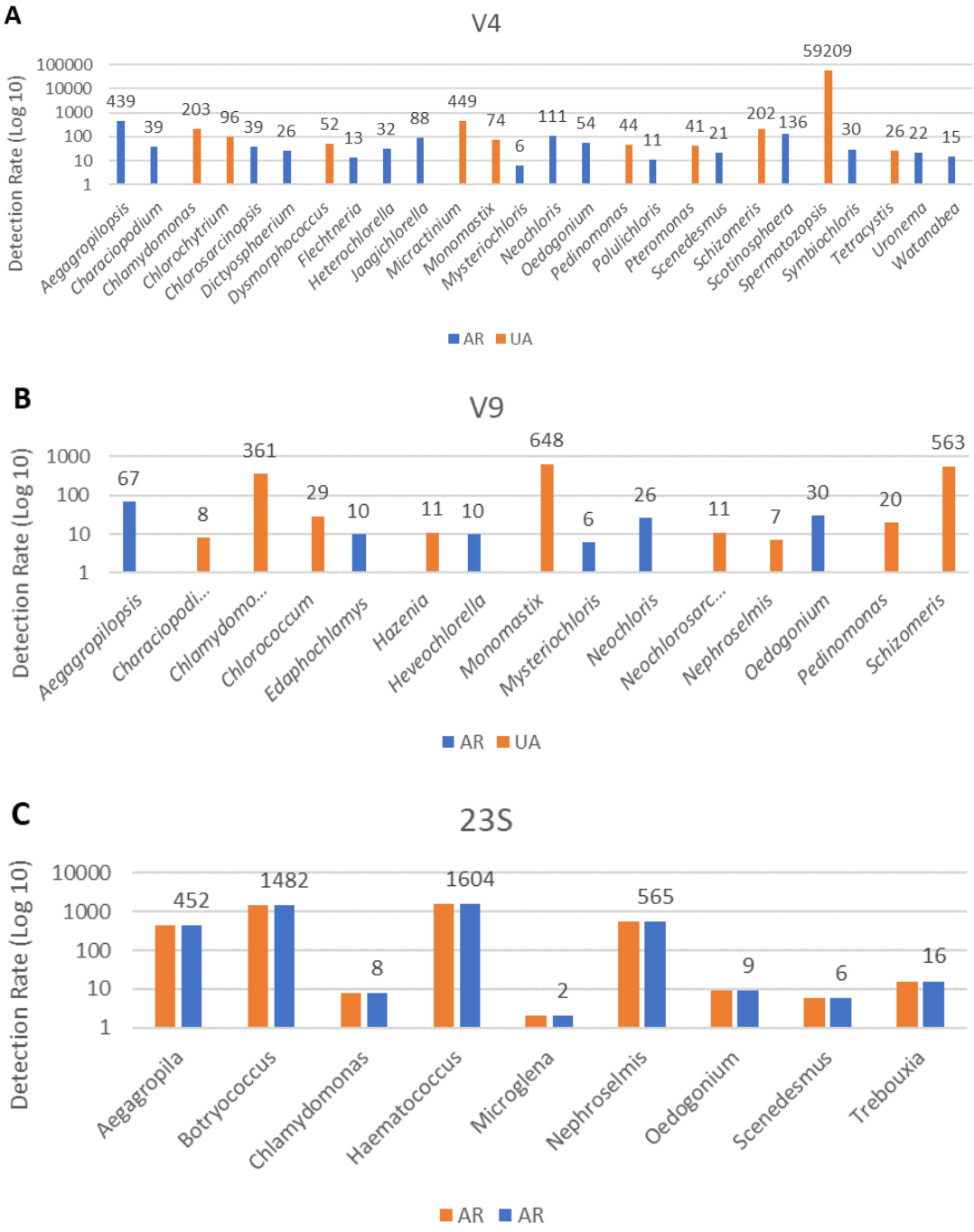


**Figure 1.** Chlorophyte genera detected by each marker. The Y-axes represent the detected ASVs, converted to logarithmic (log 10) scale to better visualise less represented taxa. The absolute number of matched ASVs for each genus is displayed atop their corresponding bar. Blue bars represent taxa found in the Arboretum, while orange bars represent taxa found near the University Avenue.
**A.** Chlorophyte genera detected and classified by V4 marker.
**B.** Chlorophyte genera detected and classified by V9 marker.
**C.** Chlorophyte genera detected and classified by 23S marker. This marker was only used in the Arboretum.

V4 marker (Table 2).

The V4 marker is the most productive for both eukaryotic surveys in general and for algal classification in particular, detecting nearly 1700 eukaryotic ASVs and classifying 26 chlorophyte genera. It also has the highest Shannon H-values among three markers, even with *Spermatozopsis* excluded. Meanwhile, the V9 primers classified fewer ASVs to the genus level but still resolved 15 chlorophyte genera, while detecting a significantly greater number of eukaryotic ASVs. The results presented here are consistent with previous studies (Tragin et al. 2018; Piredda et al. 2017) which found that the V4

primer provided better taxonomic resolution to genus level than V9. However, the latter classified more eukaryotic ASVs at higher taxonomic levels. These findings are incontrast to the results of Bradley et al. (2016), who found that the V9 primers better captured the community composition of mock samples than the V4 primers.

**Table 1.** Chlorophyte genera detected by each marker.

| | V4 | | V9 | | 23S |
|---|---|---|---|---|---|
| | AR | UA | AR | UA | AR |
| *Aegagropila* | | | | | ✓ |
| *Aegagropilopsis* | ✓ | | ✓ | | |
| *Botryococcus* | | | | | ✓ |
| *Characiopodium* | ✓ | | | ✓ | |
| *Chlamydomonas* | | ✓ | | ✓ | ✓ |
| *Chlorochytrium* | | ✓ | | | |
| *Chlorococcum* | | | | ✓ | |
| *Chlorosarcinopsis* | ✓ | | | | |
| *Dictyosphaerium* | ✓ | | | | |
| *Dysmorphococcus* | | ✓ | | | |
| *Edaphochlamys* | | | ✓ | | |
| *Flechtneria* | ✓ | | | | |
| *Haematococcus* | | | | | ✓ |
| *Hazenia* | | | | ✓ | |
| *Heterochlorella* | ✓ | | ✓ | | |
| *Jaagichlorella* | ✓ | | | | |
| *Micractinium* | | ✓ | | | |
| *Microglena* | | | | | ✓ |
| *Monomastix* | | ✓ | | ✓ | |
| *Mysteriochloris* | ✓ | | ✓ | | |
| *Neochloris* | ✓ | | ✓ | | |
| *Neochlorosarcina* | | | | ✓ | |
| *Nephroselmis* | | | | ✓ | ✓ |
| *Oedogonium* | ✓ | | ✓ | | ✓ |
| *Pedinomonas* | | ✓ | | ✓ | |
| *Polulichloris* | ✓ | | | | |
| *Pteromonas* | | ✓ | | | |
| *Scenedesmus* | ✓ | | | | ✓ |
| *Schizomeris* | | ✓ | | ✓ | |
| *Scotinosphaera* | ✓ | | | | |
| *Spermatozopsis* | | ✓ | | | |
| *Symbiochloris* | ✓ | | | | |
| *Tetracystis* | | ✓ | | | |
| *Trebouxia* | | | | | ✓ |
| *Uronema* | ✓ | | | | |
| *Watanabea* | ✓ | | | | |
| Total taxa detected | 16 | 10 | 6 | 9 | 9 |

The lower resolution in V9 may derive from a less reliable database (Piredda et al. 2017).This study used the SILVA database for both 18S markers, specifically version 132 (Callahan 2018). Unfortunately, this database is no longer updated, having been published in 2018. The latest version of the SILVA database, v138.1, is no longer appropriate for use with eukaryotes

(McLaren & Callahan 2021). A more up-to-date database might have resulted in different classification rates for both V4 and V9.

**Table 2.** Shannon diversity values (H-values) for each marker and site.

| Marker | AR | UA |
|---|---|---|
| V4 | 2.090 | 0.132(1.807[1]) |
| V9 | 1.479 | 1.305 |
| 23S | 1.308 | N/A[2] |

The 23S primer, being designed specifically for chlorophytes, detected over a thousand Chlorophyte ASVs though only 102 ASVs were classified to genus level. This marker found the least number of chlorophyte genera, but with the caveat that it was only used in one site. The primer possibly has greater utility for metabarcoding of Chlorophyta and other algal groups, given its universality among organisms with plastids and its relatively short length of around 400 base pairs that make it suitable for Illumina high-throughput sequencing. Notably, over half of the nine genera detected by this marker were not found by the 18S markers, suggesting that targeted markers should be used to complement more universal markers that might be too conserved to differentiate lower taxonomic levels (Bradley et al. 2016; Hajibabaei et al. 2019).

Unfortunately, the available database for the 23S marker is currently limited. A search on GenBank using the keywords indicated earlier returned only 768 results, with two additional sequences from complete chloroplast genomes manually added; moreover, no publicly available sequences in the BOLD. The taxonomic coverage of the GenBank sequences also leaves much to be desired, with 51 labelled as "uncultured *Trebouxia*" and another 133 representing only four species. Expanding the database of 23S sequences could increase the utility of the primer for environmental DNA surveys.

Some of the genera detected by metabarcodes have been detected in the Philippines based on previous biodiversity surveys, such as *Chlamydomonas*, *Cladophora*, *Haematococcus*, *Oedogonium*, *Pandorina*, and several species of *Scenedesmus* (Papa & Mamaril Sr. 2011; Baleta et al. 2016; Ederosas & Jumawan 2016; Tingson & Tamayo-Zafaralla 2018; Senados et al. 2021), lending support to the findings of the current metabarcoding workflow.

Since metabarcoding may not capture all biodiversity present at the time of sampling, and gaps may exist in the reference databases (Groendahl et al. 2017), traditional culture-based and morphological identification was also performed. Streak plating of water samples from the AR on Bold's Basal Medium detected several green microalgae genera, such as *Chlorococcum* (Figure 2A), and *Asterarcys* (Figure 2B) (Guiry & Guiry 2024). The identity of these genera was confirmed through traditional DNA barcoding using the tufA marker. *Asterarcys* was not detected by metabarcoding, while streak plating did not capture many of the algal genera detected using eDNA. Thus, both genetic and traditional identification methods should be considered in future Philippine algal surveys.

The results of this study demonstrate the potential of metabarcoding for studying Philippine freshwater green microalgae and identifying taxa for biological surveys. The V4 and V9 regions of the 18S gene show promise as markers for rapid biodiversity surveys of eukaryotes using environmental DNA, including Philippine chlorophytes, withas both have detected and classified over a dozen green algae genera detected and classified by each marker. The 23S marker was not tested on other eukaryotes, and its utility for chlorophytes remains somewhat limited due to a relatively small database compared to 18S genes. However, expanding the available reference sequences in Gen-

Bank and other databases, particularly those from Philippine taxa, might make this a more attractive marker in the future, given its reliable amplification in this study. Due to the varied taxa detected by each marker, a multi-marker approach might work best for Philippine metabarcoding studies (Bradley et al. 2016; Hajibabaei et al. 2019).

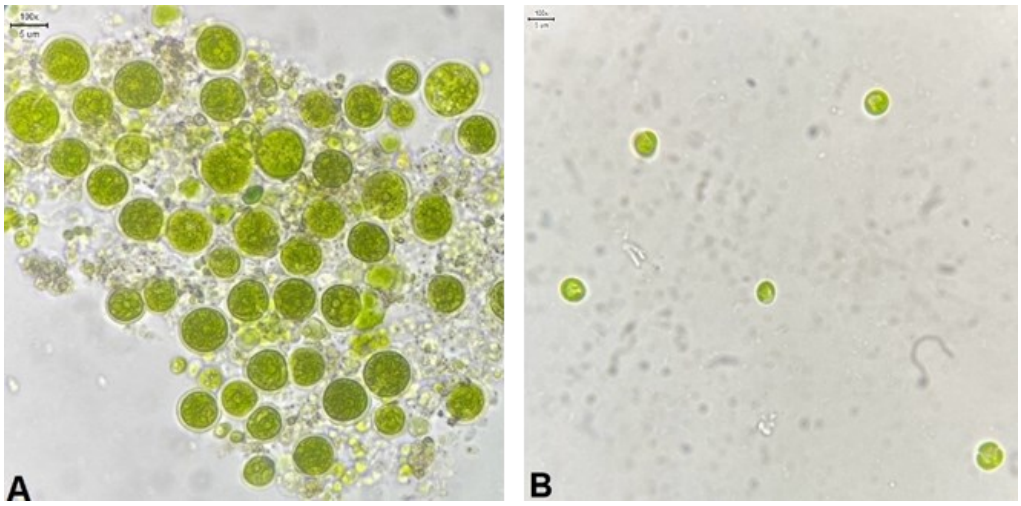


**Figure 2.** Algae cultured from water samples, viewed at 100X magnification. **A.** Clusters of unicellular *Chlorococcum* sp. isolated from the University Avenue sampling site. Scale bar = 5 µm. **B.** Isolates of unicellular *Asterarcys* sp. from the Arboretum sampling site. Scale bar = 5 µm.

## AUTHOR CONTRIBUTION

G.C.L.Q., L.R.S.S., and W.D.M. conceptualised the study. L.R.S.S. and W.D.M. supervised the research. G.C.L.Q. and W.E.P. collected the data. G.C.L.Q. analysed the data and drafted the initial version of the manuscript. All authors revised and approved the final version of the manuscript.

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## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest related to their participation in this study.

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