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Periphyton diversity in two different Antarctic lakes assessed using metabarcoding

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Abstract: Antarctic lakes have generally simple periphyton communities when compared with those of lower latitudes. To date, assessment of microbial diversity in Antarctica has relied heavily on traditional direct observation and cultivation methods. In this study, sterilized cotton baits were left submerged for two years in two lakes on King George Island and Deception Island, South Shetland Islands (Maritime Antarctic), followed by assessment of diversity by metabarcoding using high-throughput sequencing. DNA sequences of 44 taxa belonging to four kingdoms and seven phyla were found. Thirty-six taxa were detected in Hennequin Lake on King George Island and 20 taxa were detected in Soto Lake on Deception Island. However, no significant difference in species composition was detected between the two assemblages (Shannon index). Our data suggest that metabarcoding provides a suitable method for the assessment of periphyton biodiversity in oligotrophic Antarctic lakes.

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Key words: Deception Island, high-throughput sequencing, internal transcribed spacer, King George Island

Introduction

Antarctica hosts some of the most pristine environments on Earth, as well as experiencing some of the planet's most extreme conditions (Convey *et al.* 2014). Such extreme conditions act as environmental filters (Gonçalves *et al.* 2012). Antarctic lakes are an example of one such extreme environment, often being shallow (< 10 m deep) and typically cold, highly transparent (allowing penetration of high levels of solar radiation) and, in many cases, oligotrophic (Butler *et al.* 2000, Ogaki *et al.* 2019).

Lakes in the climatically less extreme Maritime Antarctic region usually host more complex communities than those of the continental Antarctic and often contain more abundant microcrustacean populations (Butler *et al.* 2000). Typically, continental Antarctic lakes are dominated by photosynthetic and heterotrophic protozoa and a limited number of algae, fungi, bacteria and viruses. Antarctic lakes have a trophic structure that is simple when compared with those of lower latitudes, and energy and nutrient flows are generally dominated by the microbial loop (Laybourn-Parry & Pearce 2007). Lakes of the Maritime Antarctic islands, as exemplified by studies on Signy Island (South Orkney Islands; Butler *et al.* 2000), have more diverse communities, but even in these metazoan diversity is low (Laybourn-Parry & Pearce 2007).

Coastal shallow lakes in the Maritime Antarctic may be subject to eutrophication through the activities of marine vertebrates (e.g. seals and penguins), leading to increased biomass (Izaguirre *et al.* 2020). Algal diversity is lower than in other parts of the world, although, as is often the case in studies of other microbial groups, the recent application of molecular approaches is starting to reveal higher levels of biodiversity than previously recognized based on traditional morphological techniques (Izaguirre *et al.* 2020). Protozooplankton or metazooplankton are the top predators in Antarctic lakes (Laybourn-Parry & Pearce 2007). Lacustrine protozooplankton comprise heterotrophic flagellates and ciliates (Izaguirre *et al.* 2020). Representatives of these groups belonging to the genera *Brachionus*, *Halteria*, *Notholca*, *Keratella*, *Lepadella*, *Hartmannella*, *Vannella* and *Vexillifera* have been reported in the freshwater zooplankton of the Antarctic Peninsula, South Shetland Islands and South Orkney Islands (Butler *et al.* 2000, Hansson *et al.* 2012). Periphyton, which can be defined as a mixture of autotrophic and heterotrophic organisms embedded in an organic matrix (Peter & Lodge 2009), is usually poorly studied overall (Hansson 1992), and very few studies to date have focused on periphyton

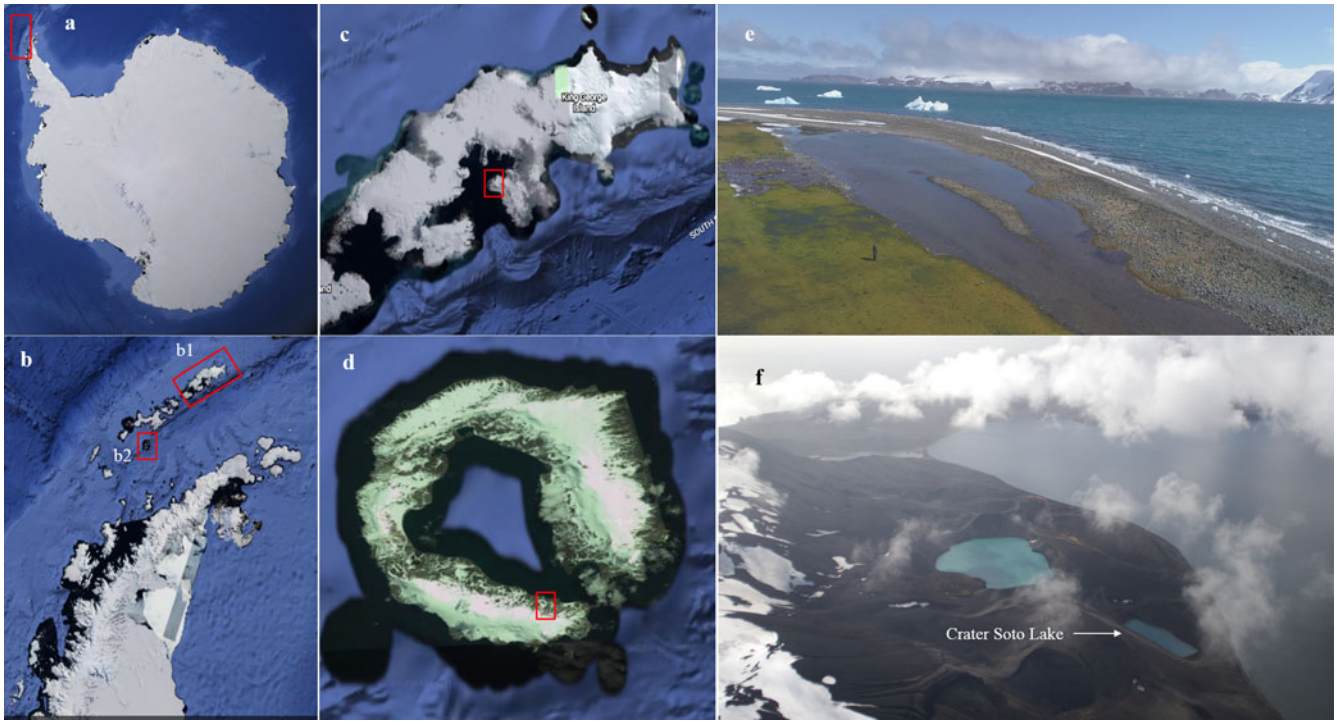

Fig. 1. a.–d. Satellite images (obtained in Google Earth Pro, 2019) and the sites where the cotton baits were sampled. **a.** Antarctic continent with the Antarctic Peninsula inside the red rectangle. **b.** Antarctic Peninsula with the South Shetland Islands archipelago; red rectangles represent King George Island (b1) and Deception Island (b2). **c.** King George Island and **d.** Deception Island, with Hennequin Point and Soto Lake indicated, respectively, within each red rectangle. **e.** Hennequin Lake (62°07'22.9"S, 58°23'46.1"W) and **f.** Soto Lake (62°58'52.0"S, 60°39'52.9"W). Pictures taken by the authors (L.H. Rosa and P.E.A.S. Câmara).

communities (Wood *et al.* 2012), especially in Antarctic lakes (Hansson 1992, Pizarro *et al.* 2002). The absence of substrata such as macrophytes, where periphyton can attach, combined with the typically low nutrient availability make such studies more challenging (Hansson 1992). Jungblut *et al.* (2005) studied periphytic cyanobacteria in ponds in the Victoria Land Dry Valleys, and Pizzaro *et al.* (2002) used artificial baits to investigate periphytic algal communities in the lakes in Hope Bay.

Assessment of microbial diversity in Antarctic lakes has, until recently, relied largely on traditional microbiological culture approaches or direct observation (Izaguirre *et al.* 2006, Gonçalves *et al.* 2012, Ogaki *et al.* 2020). However, under extreme environmental conditions, many microbes can display considerable morphological variation (Huss *et al.* 1999), hampering identification. These traditional approaches also cannot reliably detect quiescent forms (Ruppert *et al.* 2019).

Recent advances in molecular biology have provided important new tools for assessing microbial biodiversity. The use of DNA metabarcoding approaches provides an effective method for the detection of rare species (Ruppert *et al.* 2019, Câmara *et al.* 2020). However, as yet, few studies have applied metabarcoding approaches in studies of Antarctic ecosystems (but see Fraser *et al.* 2018, Câmara *et al.* 2020, Garrido-Benavent *et al.* 2020, Rosa *et al.* 2020, Ogaki *et al.* 2021). Rippin *et al.* (2018) reported that this approach revealed ~11 times greater richness than a traditional morphological approach in a study on Livingston Island, South Shetland Islands. Fraser *et al.* (2018) used metabarcoding to investigate the molecular diversity of soils in Victoria Land. Garrido-Benavent *et al.* (2020), in another study on Livingston Island, investigated the successional patterns of microorganisms, while Câmara *et al.* (2020) used metabarcoding to investigate Chlorophyta diversity in soil samples from two sites on Deception Island. In the current study, we used DNA metabarcoding and scanning electron microscopy (SEM) to assess the diversity of periphyton communities present in two lakes in the South Shetland Islands.

Materials and methods

Study sites and sampling

Sample collection followed the procedures described by de Souza *et al.* (2021). The two lakes sampled in this study during the summer (December 2016) were Soto Lake located on Deception Island (62°58'52.0"S, 60°39'52.9"W) and Hennequin Lake located on King George Island (62°07'22.9"S, 58°23'46.1"W) (Fig. 1).

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Both islands are located in the South Shetland Islands, Maritime Antarctic.

Three nylon bags (6 × 6 cm), each containing 20 cm of sterilized cellulose baits (thick cotton strings), were placed at two sites at least 20 m apart in both Hennequin Lake and Soto Lake on 1 and 10 December 2016, respectively (a total of six bags per lake). The bags were fixed by a rope attached to the lake margin; this allowed the bag to freely float on the water column up to 2 m deep (de Souza *et al.* 2021). The baits remained suspended in the water column for two years, providing matrices for colonization by freshwater organisms. In the summer of 2018/2019, all baits were collected and placed into individual sterilized Whirl-Pak bags (Sigma-Aldrich, USA), which were sealed and kept at -20°C in a sterilized box until transportation to the laboratory at the Federal University of Minas Gerais, Brazil. There, the three samples from each lake were processed to extract the total DNA. The physical and chemical parameters (temperature, conductivity and pH) of each lake were measured at each site when the baits were deployed using a Hanna HI 9828 multi-parameter probe (Hanna Instruments, USA).

DNA extraction, amplification and sequencing

The three cotton baits from each point within a lake (segments of 1 cm in length from each deployment site point) were processed together in the same DNA extraction in order to increase DNA yield, resulting in a total of two DNA samples for each lake (four in total; see also de Souza *et al.* 2021). Total DNA was extracted from environmental samples using the QIAGEN DNeasy PowerLyzer PowerSoil Kit, following the manufacturer's instructions. Extracted DNA was used as a template for generating polymerase chain reaction (PCR) amplicons. The internal transcribed spacer 2 (ITS2) of the nuclear ribosomal DNA was used as a DNA barcode for molecular species identification (Chen *et al.* 2010, Richardson *et al.* 2015). ITS2 has been widely used in barcoding studies of many organisms (Ruppert *et al.* 2009) and has proved effective in recent studies of Antarctic diversity (Câmara *et al.* 2020, Rosa *et al.* 2020, Ogaki *et al.* 2021). PCR amplicons were generated using the universal primers ITS3 and ITS4 (White *et al.* 1990) and were sequenced by high-throughput sequencing (HTS) at Macrogen, Inc. (South Korea) on an Illumina MiSeq sequencer (3 × 300 bp) using the MiSeq Reagent Kit v3 (600-cycle) following the manufacturer's protocol.

Data analyses and taxa identification

Two databases were used in the identification of the amplicon sequences obtained: the PLANiTS2 database for Viridiplantae (Banchi *et al.* 2020) and the UNITE eukaryote ITS database version 8.2 (Abarenkov *et al.* 2020) for all eukaryote groups. Raw fastq files were filtered using *BBDuk* version 38.34 (BBMap; https://sourceforge.net/projects/bbmap/) to remove Illumina adapters, known Illumina artefacts and the PhiX Control v3 Library. Quality read filtering was carried out using *Sickle* version 1.33-q 30-l 50 (https://github.com/najoshi/sickle) to trim 3' or 5' ends with low Phred quality score, and sequences < 50 bp were also discarded. The remaining sequences were imported into *QIIME2* version 2019.10 (https://qiime2.org/) for bioinformatics analyses (Bolyen *et al.* 2019).

The *qiime2-dada2* plugin is a complete pipeline that was used for filtering, de-replication, turn paired-end fastq files into merged and to remove chimeras (Callahan *et al.* 2016). It is important to note that the ranking and correct identification of taxa rely heavily on the quality and reliability of the consulted databases, so the taxonomic assignments were determined for amplicon sequence variants (ASVs) using the *qiime2-feature-classifier* (Bokulich *et al.* 2018) *classify-sklearn* against the UNITE ITS database version 8.2 (Abarenkov *et al.* 2020) and trained with a naïve Bayes classifier with a confidence threshold of 98.5%. For assessing the obtained sequence data against the PLANiTS2 database, the pipeline was executed for merged pair-ended sequences with the following plug-ins: *vsearch join-pairs* (Rognes *et al.* 2016), *vsearch dereplicate-sequences*, *quality-filter q-score-joined* (Bokulich *et al.* 2013), *vsearch cluster-features-de-novo 97% identity limit* and *vsearch uchime-denovo*. Taxonomic assignments were determined for operational taxonomic units (OTUs) using the feature classifier (Bokulich *et al.* 2018) *classify-sklearn* against the PLANiTS2 database (Banchi *et al.* 2020) trained with a naïve Bayes classifier. As the two analyses differ in generating ASV and OTU outputs, for simplicity both are referred to as 'taxa' in the following text.

Many factors, including extraction, PCR and primer bias, can affect the number of reads obtained (Medinger *et al.* 2010) and thus lead to misinterpretation of absolute abundance (Weber & Pawlowski 2013). However, Giner *et al.* (2016) concluded that such biases did not affect the proportionality between reads and cell abundance, implying that more reads are linked with higher abundance (Deiner *et al.* 2017, Hering *et al.* 2018). Therefore, for comparative purposes we used the number of reads as a proxy for relative abundance.

Ecological diversity analysis

The following diversity statistics were calculated to assess α diversity: Fisher's α, Shannon, Margalef, Simpson and evenness. We also performed a diversity *t*-test for comparison of the Shannon and Simpson diversities in

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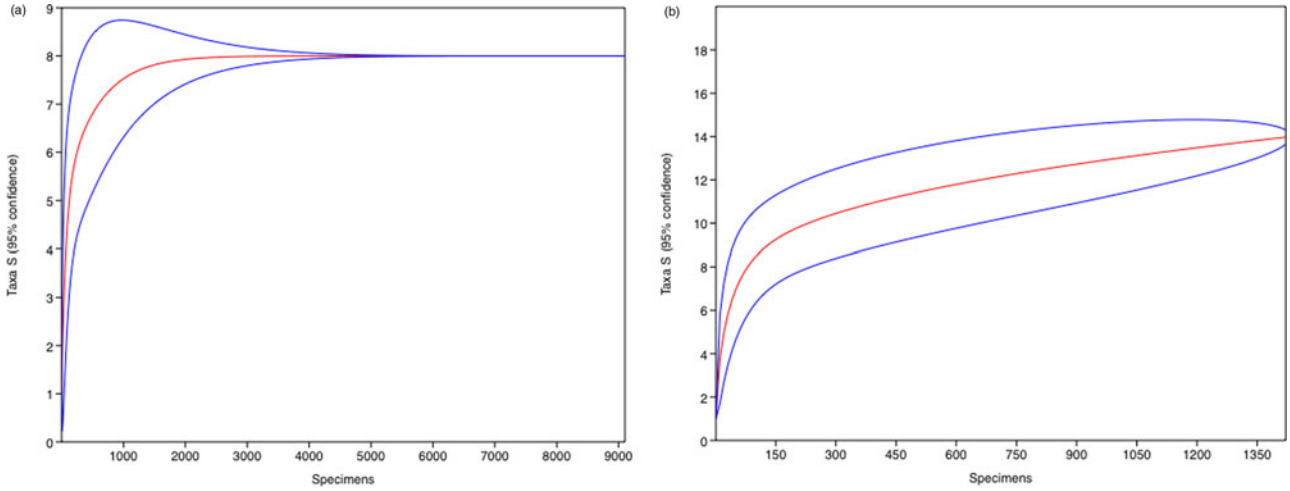

Fig. 2. Rarefaction curves based on taxa profile (0.03 similarity) from Hennequin Lake (left) and Soto Lake (right). Blue lines represent 95% confidence limits.

both lakes. All indices were calculated using *PAST* 3.26 (Hammer *et al.* 2001). Venn diagrams were prepared as described by Bardou *et al.* (2014). Rarefaction calculations were carried out using the rarefaction analysis command in the software *MOTHUR*, where we clustered sequences into OTUs by setting a 0.03 distance limit. Algal distribution is based on AlgaeBase (http://www.algaebase.org). Classification follows Leliaert *et al.* (2012) for Viridiplantae and Cavalier-Smith (2007) for Chromista, Protista and Metazoa.

Scanning electron microscopy

For SEM, subsamples of the cotton baits were fixed in 2% glutaraldehyde in 0.1 M $NaPO_4$ buffer and washed in buffered 1% OsO_4 for 2 h. The material was dehydrated using a graded ethanol series (10%, 25%, 40%, 60%, 75%, 85%, 95% and 100%) for 15 min per concentration. The material was dried in a critical point drying apparatus, sputter-coated with gold and viewed with a Quanta 200 SEM (FEI, USA).

Results

Taxonomy and diversity

The calculated rarefaction curves indicate that the sampling effort was sufficient to represent the species analysed in the two sampled lakes (Fig. 2). In total, 991,102 paired-end sequencing reads were recovered, of which 370,406 reads remained after filtering. The Eukaryota UNITE analyses identified a total of 11 taxa and the Viridiplantae PLANiTS2 analyses identified a total of 35 taxa (Table I).

DNA sequences representing 44 distinct taxa were detected in the samples from four kingdoms (Metazoa, Protozoa, Chromista and Viridiplantae) and six phyla (Arthropoda, Choanoflagellata, Ciliophora, Percolozoa, Chlorophyta and Streptophyta). A total of 36 taxa were detected in Hennequin Lake and 20 taxa were detected in Soto Lake, with 12 taxa being present in both lakes (Fig. 3). The highest diversity was represented by Chlorophyta (green algae) with 30 taxa, followed by Ciliophora, with seven taxa. Using reads as a proxy for abundance, the most abundant were *Chlorococcum microstigmatum* and *Planophila* sp. (Chlorophyta). Among the ciliates, *Cyrtohymena* sp. was the most abundant. The SEM images (Fig. 4) obtained from Soto Lake baits clearly show the characteristic silica shells of diatoms (Bacillariophyta). The SEM images are consistent with genera such as *Gomphonema* sp. and *Synedra* sp., which are both common and cosmopolitan taxa.

The α diversity indices are presented in Table II. The diversity *t*-test for the Shannon and Simpson indices were not significant, indicating no statistical differences in species compositions between the two assemblages.

Discussion

Reported algal diversity in Antarctic lakes includes chlorophytes, chrysophytes, cryptophytes, diatoms, dinoflagellates and prokaryotic cyanobacteria (Mataloni & Pose 2001, Lizotte 2008, Izaguirre *et al.* 2020). Algal diversity in these lakes illustrates the influence of the surrounding environment, such as in the presence of *Raphidonema* spp. blooms that derive from melting snowfields. Aerophilic taxa such as *Prasiola crispa* (Lightfoot) Kützing, 1943, which can show macroscopic growth in the margins of eutrophic water bodies, could also be present in lake samples in this region.

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Table I. Taxa present in Hennequin Lake and Soto Lake as indicated by sequence data obtained. Geographical distribution: A = Antarctica, Ae = Antarctic endemic, Ar = Arctic, As = Asia, Au = Australia, B = bipolar, C = China, E = Europe, I = India, NZ = New Zealand, SA = South America, W = cosmopolitan. Habitat: A = aerial, C = chasmoendolithic, F = freshwater, M = marine, NA = North America, S = snow, T = terrestrial, U = ubiquitous. Taxa at the genus level may be missing habitat or geographical distribution data. Metazoa are based on the UNITE database and Viridiplantae on the PLANiTS2 database.

Taxa	Distribution	Habitat	Number of DNA reads	
			Hennequin Lake (King George Island)	Soto Lake (Deception Island)
Kingdom Metazoa				
Phylum Arthropoda				
Folsomia sp.	W	T	32	0
Kingdom Protozoa				
Phylum Choanoflagellata				
Choanoflagellida	W	M/B/F	5	18
Phylum Percolozoa				
Allovahlkampfia sp.	W	T/F	8	18
Kingdom Chromista	W	M/B/F	8	452
Phylum Ciliophora				
Bryophyllum sp.	E, NA, A	T/F	13	0
Halteria sp.	W	F	20	0
Oligohymenophorea	W	M/F	3	0
Oxytrichidae	W	M/F/T	71	0
Cyrtohymena sp.		M	139	366
Stylonychia sp.	W	T/F	11	0
Urostylida	W	M/F/T	173	0
Kingdom Viridiplantae				
Phylum Chlorophyta				
Chlorophyceae	W	M/T/F	1	0
Chlamydomonadales			57	1
Chlamydomonas nivalis (F.A. Bauer) Wille	W	S	0	2
Chlamydomonas proboscigera Korshikov	E, As	T/F	2	0
Chlamydomonas raudensis Ettl.	E, A	F	0	11
Chlamydopodium starrii (Fott) Ettl & Gärtner	Ar, A, E, Af	T	0	1
Paulschulzia pseudovolvox (P.Schultz) Skuja	E, AS, As, Au	F	11	0
Tetracystis sp.	E, NA	T/F	3	0
Chlorellales				
Chlorococcum microstigmatum P.A.Archibald & Bold	E, NA	T	1525	0
Chlorococcum sp.	E, NA	T/F	54	2
Chloromonas fonticola (R. Brabez) Gerloff & Ettl		F	7	0
Jaagichlorella luteoviridis (Chodat) Darienko & Pröschold	Ar,E,NA,Af,As	F	8	0
Micractinium sp.	W	F	41	0
Prasiolales				
Desmococcus olivaceus (Persoon ex Acharius) J.R. Laundon	W	A/T/C	2	0
Koliella longiseta (Vischer) Hindák	E	F	90	24
Prasiola sp.	W	F/T	12	0
Raphidonema nivale Lagerheim	W	F	1	0
Sphaeropleales				
Chodatodesmus australis Sciuto, Verleyen, Moro & La Rocca	Ae	T/F	12	0
Coenochloris signiensis (Broady) Hindák	A, E	T	23	0
Mychonastes sp.	NA, E, AS	M/F	2	12
Neocystis sp.	E	F	0	1
Ulotrichales				
Chlorothrix sp.	E	M	12	10
Planophila bipyrenoidosa Reisigl	E	T	3	1
Planophila sp.	E	T	965	174
Pseudendoclonium sp.	W	M/F	0	7
Ulothrix sp.	W	M/F	0	81
Trebouxiales				
Coccomyxa sp.	E		2	0
Trebouxiophyceae	W	A/T	1	0
Trebouxia asymmetrica Friedl & Gärtner	E	T	6	29
Trebouxia aff. *decolorans*	E	T	0	5
Clade Streptophyta				
Sanionia sp.	B	T/F	1	0
Liliopsida	W	T	3	0
Raphanus sp.	W	T	0	1

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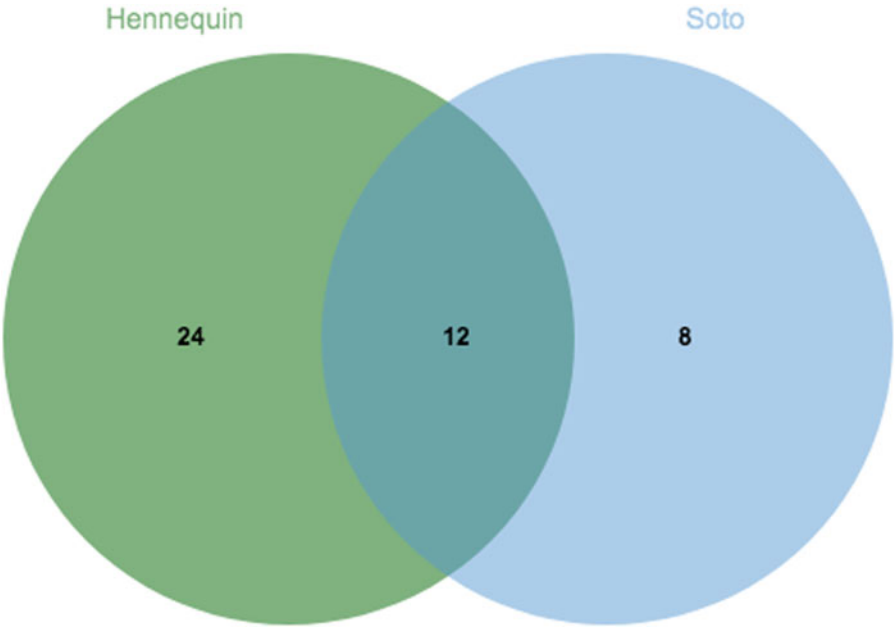

Fig. 3. Venn diagram of taxa that are unique or shared between Hennequin Lake (green) and Soto Lake (blue).

The sampled lakes differ in many features, including depth, water temperature and proximity of surrounding vegetation. Hennequin Lake is a shallower lake and is slightly warmer, as well as being surrounded by vegetation. Based on the sequence data obtained, it hosts greater diversity than Soto Lake, which is a much deeper and colder lake with much more limited vegetation in its catchment.

The only sequence of Arthropoda found was a Collembola, referred to the genus *Folsomia*, in Hennequin Lake, which is much closer to moss carpets (Fig. 1). According to Greenslade (1995), 11 species of springtails are native to the Maritime Antarctic. However, members of *Folsomia* are not native to this region. The parthenogenetic non-native species *Folsomia candida* Willem 1902 has been recorded from Deception Island,

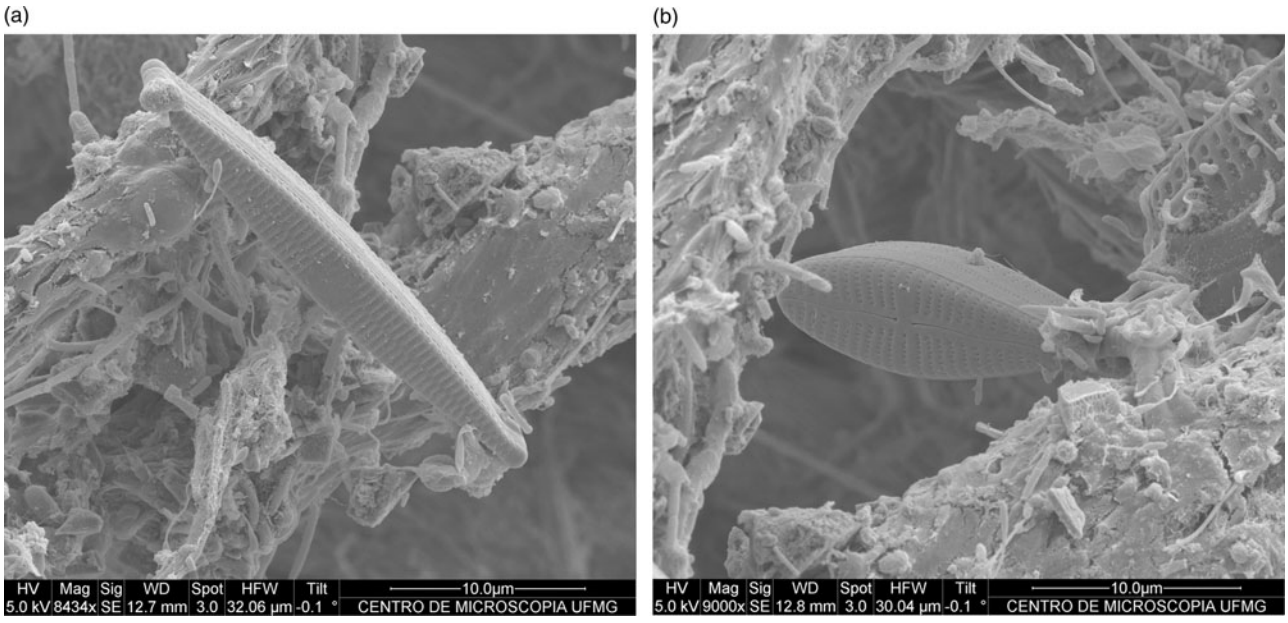

Fig. 4. Scanning electron micrographs showing the diatoms *Synedra* sp. (left) and *Gomphonema* sp. (right) attached to cotton baits obtained from Soto Lake, Deception Island.

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Table II. Diversity indices of assemblages from Hennequin Lake and Soto Lake and physical and chemical lake parameters.

Indices and physical and chemical parameters	Hennequin Lake (King George Island)	Soto Lake (Deception Island)
Taxa	36	20
Fisher's α	5.64	3.40
Simpson	0.69	0.74
Shannon	1.73	1.70
Evenness	0.15	0.27
Margalef	4.31	2.67
Temperature (°C)	6.5	4.9
Conductivity (mS cm^{-1})	105	313
pH	9	9.2

but its continued presence or status there is uncertain (Greenslade *et al.* 2012). Choanoflagellida have been reported in the Antarctic marine ecosystem of the Weddell Sea (Buck & Garrison 1988), while members of this class are also known to occur in freshwater habitats elsewhere (Leadbeater & Kelly 2001); however, to our knowledge, this is the first such report from Antarctic lakes.

The sequence data representing Chromista could only be resolved to higher taxonomic rank; however, they were dominant in Soto Lake, and the SEM data were useful in confirming this. The SEM data also confirmed the presence of two diatoms, which were not detected in the metabarcoding analysis. Among the ciliates, Hennequin Lake again hosted a more diverse community than Soto Lake. Few studies have addressed this diverse group in Antarctica, and it is likely to have been overlooked previously. Percolozoa have a cosmopolitan distribution, including reports from extreme environments (Park & Simpson 2011), and they are another group that is likely to have been overlooked.

The Viridiplantae sequence diversity identified here resembles that described by Câmara *et al.* (2020) from Deception Island. However, marine taxa were not detected, even though both lakes sampled are located relatively close to the shore. Some taxa (such as Trebouxiales) are commonly present in the airspora, from which they can be deposited into freshwaters. DNA of the moss genus *Sanionia* was also detected in Hennequin Lake. This is one of the most common and widespread moss genera in moist and waterlogged habitats in the Maritime Antarctic, with large carpets surrounding Hennequin Lake. Concerning the flowering plant sequences identified, the record of Liliopsida may refer to the Antarctic hairgrass (*Deschampsia antarctica* E. Desv.), which is also common in the vicinity of Hennequin Lake. The presence of DNA attributed to a radish (*Raphanus* sp.) in Soto Lake has no clear explanation, although it provides a further example that, if confirmed, indicates that DNA (at least) of non-native species can be transferred into Antarctica (e.g. Fraser *et al.* 2018) by means of either air transport or zoochory or by humans in the form of plant fragments, propagules, pollen or even single cells (see also similar reports of non-native assigned sequences in Câmara *et al.* 2020, Rosa *et al.* 2020). Both lakes studied here are close to areas of human activity, with Hennequin Lake being located close to an Ecuadorian refuge and Soto Lake being near the Spanish station Gabriel de Castilla, while Deception Island is one of the most visited tourist sites in Antarctica (Câmara *et al.* 2020).

It is interesting that, apart from the mentioned lake differences, no statistical differences in species compositions between the two assemblages were found. This needs further investigation, but the fact that both lakes are subject to the same harsh environmental conditions and low nutrient availability and that both lakes are located in the same geographical region of the Maritime Antarctic could play a role.

Few studies are available against which to compare our data. Pizzaro *et al.* (2002), working on periphyton communities in three lakes in Hope Bay, reported 18 Chlorophyta taxa, 12 of which were only assigned to the genus level, whereas we identified 28 chlorophytes, with 16 at the species level. As further metabarcoding studies are completed and database coverage progressively improves, considerable increases in recorded diversity are likely to result in all groups.

Conclusions

This study is the first application of metabarcoding in the assessment of periphyton diversity in Antarctic lakes. We recognize that, as in all metabarcoding studies, detecting the presence of DNA sequences referred to a given taxon does not confirm the presence of viable material, while sequence identification remains strongly limited by the available databases. We suggest that application of an integrated approach including both traditional and metabarcoding methodologies is required to generate the most authoritative and reliable data. Accepting these caveats, the DNA sequence data obtained in the current study are consistent with the presence of a rich periphyton community in these environmentally harsh Antarctic lakes. The application of metabarcoding yielded a greater diversity than has been previously recognized through traditional morphological approaches, supporting the use of metabarcoding as a powerful tool for studying periphyton communities in Antarctic lakes.

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Author contributions

PEASC, PC, MC-S and LHR designed the study. OHBP performed the bioinformatics. LMDS, ETA, LHR, MC-S and LHR conducted the fieldwork. LMDS performed the scanning electron microscopy analysis and lab work. ETA conducted the ecological analyses. PEASC and LHR secured funds. All authors wrote and revised all of the versions of this manuscript.

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