Estuarine, Coastal and Shelf Science 319 (2025) 109284

Contents lists available at ScienceDirect

Estuarine, Coastal and Shelf Science

journal homepage: www.elsevier.com/locate/ecss

Eukaryote biodiversity in supratidal microbialite pools: A foundational environmental DNA assessment

Arsalan Emami-Khoyi [b,a,1], Claudia M. Schnelle [a,1], Dave R. Clark [c], Silke Laakmann [d,e], Peter R. Teske [a,f], Gavin M. Rishworth [f,g,*]

[a] Centre for Ecological Genomics and Wildlife Conservation, Department of Zoology, University of Johannesburg, Auckland Park, South Africa
[b] Institute for Wildlife Management and Nature Conservation, Hungarian University of Agriculture and Life Sciences, Gödöllő, Hungary
[c] School of Life Sciences, University of Essex, Colchester, United Kingdom
[d] Helmholtz Institute for Functional Marine Biodiversity at the University of Oldenburg (HIFMB), Oldenburg, Germany
[e] Alfred-Wegener-Institute, Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany
[f] Department of Zoology, Nelson Mandela University, Gqeberha, South Africa
[g] DSI/NRF Research Chair: Shallow Water Ecosystems, Institute for Coastal and Marine Research (CMR), Nelson Mandela University, Gqeberha, South Africa

ARTICLE INFO

Keywords:
eDNA
Foundational habitat
Integrated taxonomy
Metabarcoding
Metazoans
Microphytobenthos
Stromatolites
Rocky shore

ABSTRACT

Coastlines are a mosaic of habitats, including rocky shores, sandy beaches, estuaries, and artificial substrata. Although modern microbialite pool formations were only recently discovered as an additional coastal habitat along the southern African coastline, they are now known to be surprisingly common to this region. These ecosystems function similarly to estuaries, where seawater and freshwater mix, but with groundwater as the freshwater source instead of river flow. Traditional community assessments from morphological identifications have revealed some similarities between the organisms inhabiting microbialite pools to those of nearby estuaries, but no systematic comparison has so far been undertaken. Here, we used molecular methods based on environmental DNA (eDNA) metabarcoding to characterise the eukaryote assemblages within and between three coastal southern African microbialite pools. We hypothesised that the three sites are taxonomically analogous to one another, which would support the existence of similar core ecological communities. Three genetic markers, one for metazoans (COI) and two for algae (rbcL and the V2+V3 regions of 18S rRNA) were targeted for metabarcoding. Our results show that the biodiversity of the pools was dominated by diatoms (particularly of the genera *Navicula* and *Nitzschia*) and, among the metazoans, by malacostracans, rotifers and nematodes. Although the three microbialite pools had similar broadscale community compositions at higher taxonomic levels (class and family), distinct community structure at lower taxonomic levels was observed, which may be a result of numerous opportunistic species being present in addition to the core organisms. The macroinvertebrate fauna of microbialite pools (e.g. peracarid crustaceans, polychaetes and insects) is well documented, although most are still missing from the DNA barcoding reference library. In contrast, the meiofauna (e.g. rotifers, nematodes and ostracods) is understudied. It remains unclear whether the two dominant diatom genera are the primary contributors to microbialite formation, or if other yet-undescribed species also contribute to the process. This study serves as an initial step in uncovering the hidden level of biodiversity within the unique microbialite ecosystems along the southern African coastline.

1. Introduction

Microbialites represent some of the earliest evidence of life on Earth, with a continuous fossil record dating back at least 3.43 Ga (Riding, 2010). Today, microbialite systems are relatively rare but offer valuable insights into the formative processes of those ancient habitats (Smith et al., 2011). Microbialites are biosedimentary structures formed through the precipitation of carbonate mineral forms by bacteria (Reid et al., 2000) and the trapping and binding of sediment by other microorganisms, such as cyanobacteria and diatoms (Suarez-Gonzalez et al.,

* Corresponding author. Department of Zoology, Nelson Mandela University, Gqeberha, South Africa.
E-mail address: gavinr@mandela.ac.za (G.M. Rishworth).
[1] These authors contributed equally to the study and should be considered joint first-authors on this manuscript.

https://doi.org/10.1016/j.ecss.2025.109284
Received 21 November 2024; Received in revised form 27 March 2025; Accepted 27 March 2025
Available online 28 March 2025
0272-7714/© 2025 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

2019). Modern microbialites form in a range of shallow lacustrine and marine habitats, usually characterised by extreme salinity, thermal or turbidity conditions (Rishworth et al., 2020). Microbialite-forming pools (in excess of 1000 locations identified thus far) have recently been recognised along South Africa's diverse coastline (Smith and Uken, 2003; Perissinotto et al., 2014; Rishworth et al., 2019a, 2020; Harris et al., 2025). The South African microbialite pools form at the supratidal interface of groundwater spring discharge and are globally unique, as they represent estuarine-like habitats at the interface between groundwater springs and rocky shores which are subject to periodic tidal ocean influence (Rishworth et al., 2017c, 2020). In South Africa, microbialites are formed by a consortium of microscopic 'builders', primarily cyanobacteria and diatoms, which form cohesive biofilms that can mediate carbonate lithification and can, in some cases, trap and bind sediment (Rishworth et al., 2016a; Dodd et al., 2021). It relies on conducive hydrochemical conditions (alkaline pH and high alkalinity), as well as the minimal impact of grazing and burrowing organisms that might disrupt the microbial accretion (Rishworth et al., 2016b, 2017d, 2019b, 2020). Depending on the local physical forces and underlying geology, as well as the biological pressures, spring-fed microbialites might develop clear layered (stromatolite) or clotted (thrombolite) macrostructures that develop into a suite of mesofabric types as a function of location and hydrological forces (Edwards et al., 2017; Garner et al., 2024). Under suitable conditions, repeated cycles of growth form the characteristic "barrage pools" indicative of coastal spring-fed microbialites (Forbes et al., 2010; Cooper et al., 2022).

Fauna and flora commonly observed in the region's estuaries have been identified within the microbialite pools, including diverse microalgal, macroalgal, macrophyte, invertebrate (macrofauna, >500 μm in size, only; meiofauna, 63–500 μm in size, have been comparatively understudied), and fish communities (Rishworth et al., 2017a, 2017b, 2020). Despite the small size of microbialite pools (<1–100 m^2) compared to functional estuaries (100s–1000s of m^2), many of the typical benthic macroinvertebrates occurring in nearby estuaries (Teske and Wooldridge, 2001) also represent prominent macrofauna in the microbialite pools (Rishworth et al., 2017b, 2024). In addition, microbialite pools serve as biodiversity refugia for endemic species, such as the tanaidid *Sinelobus stromatoliticus* (Rishworth et al., 2019c) or extralimital fish species such as *Coryogalops sordidus* (Rishworth et al., 2017a; du Toit et al., 2024). From a bacterial point of view, high levels of beta-diversity within and between adjacent microbialite pools has been observed in South Africa (Perissinotto et al., 2014; Rishworth et al., 2020; Waterworth et al., 2021; Neuhaus et al., 2024). The microalgal community includes marine diatoms that are washed in by tidal surges, with a persistent assemblage akin to karstic lakes and apparently globally cosmopolitan taxa (Bornman et al., 2017; Rishworth et al., 2020).

The present study is the first to assess the biodiversity of the eukaryotic community associated with supratidal spring-fed microbialite pools based on a multi-maker environmental DNA (eDNA) metabarcoding approach. This recently-developed biomonitoring tool, which uses high-throughput sequencing of environmental samples (Taberlet et al., 2018; Harrison et al., 2019), has shown great potential to overcome the limitations of conventional methods for reporting the presence of species by often identifying greater taxonomic diversity. We used eDNA metabarcoding to allow a holistic view of the eukaryote communities present in South Africa's microbialite pools, with the aim of determining whether these unique ecosystems are taxonomic analogues of each other and have a functionally similar core ecological community. This baseline molecular study can complement prior morphological community-level assessments and serves as a first step towards assessing community overlaps between distinct coastal habitats, including microbialite pools, rocky shores and estuaries. Our study shows to what extent molecular methods are suitable for describing the local flora and fauna, and where there are still gaps that can be closed in the future by improving the DNA reference databases of various local taxa.

2. Materials and methods

2.1. Study sites

The present research focusses on microbialite pools from three locations that are characteristic of the southeastern coast of South Africa (Fig. 1) and that have been well studied in other respects (Rishworth et al., 2020): a site east of Seaview (SV) (34°01′03.16″S, 25°21′56.48″E; see also Fig. 2A–C), Schoenmakerskop (SK) (34°02′28.23″S, 25°32′18.60″E; Fig. 2D) and a site west of Cape Recife (CR) (34°02′42.13″S, 25°34′07.50″E; Fig. 2E). These pools form on deformed metasediment or metagreywacke, quarzitic and phyllite bedrock from which aeolian-derived groundwater springs emerge (Edwards et al., 2017).

2.2. Sample collection and DNA extraction

Sampling was conducted in barrage pools (Fig. 2) in November 2021. Prior to sample collection, all tools were thoroughly cleaned with 10 % bleach followed by rinsing with distilled water to prevent sample DNA degradation from remnant bleach, and new gloves were worn before collection to prevent contamination between sites. At each of the three sampling pools, approximately 25–30 ml of sediment was collected from several random locations within the pools using sterile spatulas and transferred into sterile 50 ml centrifuge tubes. Samples included sediment from the bottom of each pool, as well as microbialite pieces broken off the top of smooth laminar flat mesofabric. To further avoid contamination, samples were collected from downstream to upstream locations, and researchers did not step into the pools.

Tubes were immediately placed on dry ice to prevent degradation of DNA while in the field, and subsequently stored in a freezer at −20 °C until being processed within 72 h. Prior to extraction, the tubes were kept in a refrigerator to defrost overnight, after which extractions were done in a bleach-cleaned laboratory. Samples were ground up using bleach-cleaned ceramic mortar and pestles and then separated into three replicates per site (~0.5 ml of ground sediment per tube), which were extracted individually using the DNeasy PowerSoil kit (Qiagen, Germany). Extraction of the DNA followed the manufacturer's instructions, with all extracts frozen prior to proceeding with the amplification of genetic markers.

2.3. DNA amplification

Following Taberlet et al. (2018), we consider environmental DNA to include both DNA from intact cells and extracellular genetic remains present within environmental samples, even if their identity is not fully elucidated or their presence is rare or cryptic in that habitat. To comprehensively identify the eukaryotic biodiversity, with a focus on phytobenthos and metazoans, we applied a multi-marker approach that included portions of COI, rbcL and 18S rRNA (including the variable V2 and V3 regions), on sediment samples collected from three microbialite pools located on the southeastern coast of South Africa.

Three primer pairs were used to amplify the three molecular markers (Table 1). Each of the three extraction replicates per site was amplified in a 15 μl reaction volume, followed by marker-specific PCR thermocycling. Thermocycling steps for COI included a denaturation step of 95 °C for 15 min, 13 touchdown cycles, each comprising denaturation (94 °C for 30 s), annealing (starting at 69.5 °C, with a 1.5 °C temperature reduction during each cycle for 90 s) and an extension step (72 °C for 90 s). This was followed by forty cycles with a constant annealing temperature, including denaturation (94 °C for 30 s), annealing (50 °C for 30 s) and extension (72 °C for 60 s), and concluded by a final extension step at 72 °C for 10 min. The thermocycling procedure for rbcL and 18S was the same, except for the annealing and extension times of the touchdown phase, which were 30 s and 60 s, respectively, and the touchdown procedure being followed by only 35 cycles with a constant

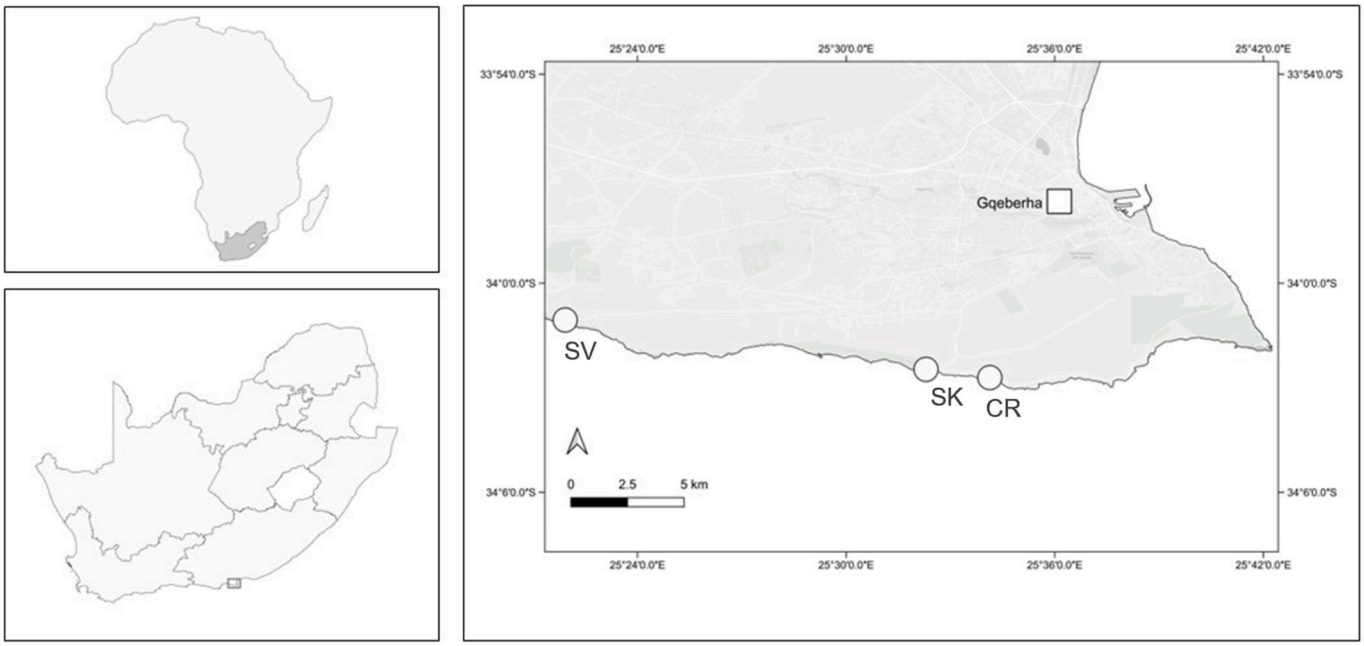

Fig. 1. The location of three supratidal microbialite pools sampled for eDNA in this study (white circles): SV (Seaview), SK (Schoenmakerskop) and CR (Cape Recife). These sites are located to the southeast of the city of Gqeberha, along a portion of South Africa's south coast where such pools are particularly abundant (for a full distribution, see Rishworth et al., 2020). The three study sites are shown with reference to the inset area in South Africa and Africa, as the map outlines on the left panels indicate.

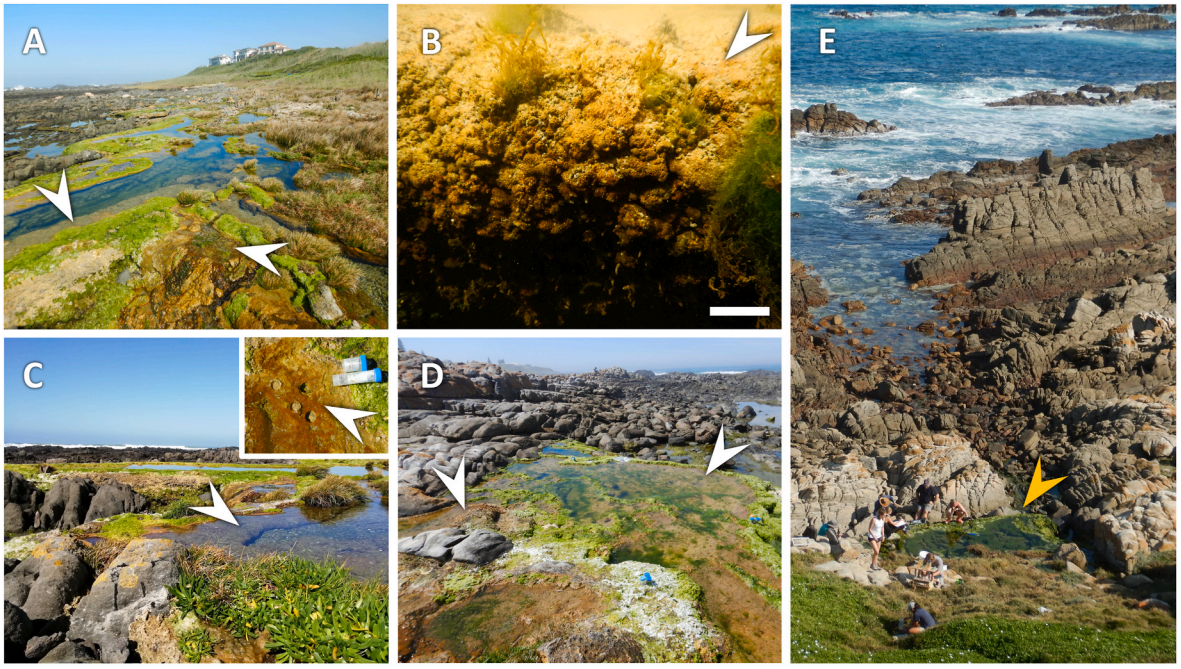

Fig. 2. Modern microbialites form in South Africa at sites where groundwater seeps discharge from the supratidal zone, forming characteristic pools that experience cyclical shifts between marine and freshwater states. In this study, microbialite pools were at Seaview (SV) (**A**–**C**), Schoenmakerskop (SK) (**D**), and west of Cape Recife (CR) (**E**). These are built by layered deposition of sediment and precipitation of calcium carbonate by a microbial and microalgal biofilm (**B**; scale bar: 2.5 cm; at SV), which was the material sampled in this study. White arrows indicate the relative position of the smooth laminar flat mesofabric that was sampled for sequencing, which is visible as an inset in panel **C**. The yellow arrow indicates the relative position of the main microbialite pool within a coastal context. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

annealing temperature of 50 °C. All PCR reactions included three positive and negative controls.

A Nextera XT DNA Library Preparation Kit (Illumina, USA) was used to create genomic libraries, which were then sequenced on an Illumina MiSeq platform (San Diego, California, USA), using 2 x 300 bp paired-end chemistry according to the manufacturer's instructions.

2.4. Bioinformatics and statistical analysis

The quality of the raw sequences was visually checked using FastQC

Table 1
Nucleotide sequences and source citations of the primer pairs used for the amplification of the three molecular markers.

Marker	Primers	Sequences (5′-3′)	Reference
COI	mlCOIintF	GGWACWGGWTGAACWGTWTAYCCYCC	Leray et al. (2013)
	jgHCO2198	TANACYTCNGGRTGNCCRAARAAYCA	Geller et al. (2013)
rbcL	BacirbcLf2	GAAGGTTTAAAAGGTGGTTTAGA	Liu et al. (2020)
	BacirbcLr2	CTACACCAGACATACGCATCC	Liu et al. (2020)
18S (V2+V3)	605F	CTGCGAACGGCTCATTAT	Liu et al. (2020)
	605R	AGGCCCGGCATTGTTATT	Liu et al. (2020)

v.0.12 (Andrews, 2010). Potential adapter contamination, as well as low quality sequences (which were defined as any four consecutive sequences with an average Phred score <20), were removed using Trimmomatic v.0.36 (Bolger et al., 2014). The presence of the forward and reverse sequencing primers was inspected in cutadapt v.4.8 (Martin, 2011), and in cases where either the forward or reverse primer was not recognised, both reads were discarded. The resulting quality-filtered sequences were further processed using VSEARCH v. 2.28.1 (Rognes et al., 2016). For this purpose, first the forward and reverse reads were merged, and only a subset of merged sequences with an expected error rate not exceeding 0.75 were selected for the downstream analyses. Of the remaining unmerged sequences, the inspection of the error profiles showed that unmerged forward sequences were consistently of higher quality compared to unmerged reverse reads, as is typical of Illumina sequencing platforms (Kwon et al., 2013; Edgar and Flyvbjerg, 2015). Thus, the unmerged forward sequences were also selected for the downstream analysis and were processed in parallel with the merged sequences. Following this step, quality filtered sequences were dereplicated into unique representative sequences and then all putative chimeric sequences were removed using the *denovo* method implemented in VSEARCH. The remaining non-chimeric sequences were clustered into unique Operational Taxonomic Units (OTUs, representative of putative species), and the centroid and the number of sequences that formed each cluster were reported. Since the clustering step is sensitive to the selection of a similarity threshold, the value for this parameter was set based on earlier studies using the same markers used in this study. The similarity threshold for 18S was set to 99 % (Giebner et al., 2020) and those of COI and rbcL were set to 97 % (Alberdi et al., 2018; Vasar et al., 2023; Liu et al., 2024).

To assign taxonomy to the reconstructed OTUs, sequences were searched against a local database of reference sequences using BLAST v.2.12 (Altschul et al., 1990) for each marker, which were downloaded from the NCBI nucleotide database in May 2024 using NCBI Entrez Direct (Kans, 2024). For each query sequence, the five best matches with a minimum percentage identity of 88 % and a minimum length of 100 bp were reported. A last common ancestor (LCA) taxonomy rank was assigned to each sequence using BASTA v.1.4 (Kahlke and Ralph, 2019). Briefly, BASTA accepts blast output files in a tabular format and assigns the taxonomic rank of reconstructed OTUs to the lowest rank shared by the reported matches. For OTUs with fewer matches, which is typical of underrepresented taxa, only the best match that satisfies selected similarity thresholds was reported. The resulting OTU and taxonomy tables were analysed using a combination of phyloseq v.1.48 (Mcmurdie and Holmes, 2013) and vegan v.2.6–6.1 in RStudio (R Core Team, 2024).

Prior to data visualisation, all datasets were bioinformatically filtered to remove sequences that matched bacteria, microscopic fungi, or that had no conclusive taxonomic match. Furthermore, since the objective of COI sequencing was to identify the diversity of invertebrates in each pool, OTUs matching algae were also removed from this dataset, and the diversity of this group of taxa was instead explored using the more specific rbcL and 18S markers. To reduce the effect of trace amounts of non-target DNA from adjacent marine, freshwater and terrestrial habitats on subsequent analyses, all taxa with a single occurrence were also removed. In order to quantify the level of biodiversity in each pool, three indices of alpha diversity at the family level were calculated in phyloseq: observed richness, i.e., the putative number of taxa present at each site (Colwell, 2009), Shannon (1948) and Simpson diversity indices (Simpson, 1949), which take into consideration taxon abundance and evenness of distribution. Kruskal-Wallis tests (Kruskal and Wallis, 1952) were conducted in the same package to determine if there were differences in a particular alpha diversity index between pools. To analyse beta diversity, the Analysis of Similarities (ANOSIM) (Clarke, 1993) method, which is implemented in the anosim2 function in the vegan package (Oksanen et al., 2024), was performed on a Bray-Curtis dissimilarity matrix (Bray and Curtis, 1957), and the results were visualised by constructing principal coordinate analysis (PCoA) ordination plots.

To investigate a potential multivariate link between environmental variables and the observed biodiversity in each microbialite pool, environmental data including water temperature, salinity, pH, oxygen saturation, dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) were extracted from a long-term environmental dataset for the three pools investigated here (Rishworth et al., 2017c). A redundancy analysis (RDA) was performed on a Hellinger transformed species abundance table (square root of relative abundance, which is bounded by 0 and 1) and standardized environmental variables (mean of 0 and SD of 1) using vegan. The statistical significance of environmental effects correlated with OTU biodiversity was tested using 9999 permutations.

3. Results

The Illumina sequencing run produced a total of 8 177 328 sequences with an average length of 292 bp. The number of sequences varied between 2 069 952 sequences for COI to 3 086 288 sequences for rbcL (Supplementary data). The presence of the primer pairs used for amplification was identified in more than 99 % of the generated sequences, confirming that all three target markers were successfully amplified. A total of 890 849, 1 383 939, and 1 332 569 sequences were processed by the VSEARCH pipeline for COI, rbcL and 18S, respectively. The pipeline collapsed both merged and unmerged forward sequences into a total of 135 942 unique sequences for COI, 2142 unique sequences for rbcL and 7510 unique sequences for 18S. The *de novo* chimera detection method classified 86.5 %, 66.2 % and 80.8 % of the unique sequences as non-chimeric for the same markers. Clustering non-chimeric sequences into OTUs resulted in the identification of a total of 2427 OTUs for COI, 113 OTUs for rbcL, and 686 OTUs for 18S.

After removal of non-target taxa from the COI dataset, the remaining OTUs were assigned to 13 classes (Table 2). Dominant classes based on relative OTU abundance of individual sequencing depths of the samples (Fig. 3A) were the Chromadorea (nematodes), Eurotatoria (rotifers), and Malacostraca (decapod crustaceans). With the exception of Enoplea, the remaining classes occurred at all sites, but at lower frequencies.

The rbcL dataset comprised OTUs assigned to only two classes, with the Bacillariophyceae being particularly important (Table 2), and an OTU assigned to the genus *Nitzschia* comprising the largest number of sequences (Fig. 3B). The 18S marker amplified a greater diversity of algae, which included an additional class of diatoms

Table 2
Target taxa identified within three supratidal spring-fed microbialite pools on the South African coast (SV: Seaview, SK: Schoenmakerskop, CR: Cape Recife) based on COI, rbcL and 18S OTU delineation. Putative identifications are based on information contained within the first or most comprehensive published accounts of each respective organism, as the references identify; several of these references have images of the taxa identified. Genetic sequences without a putative taxon associated either have not been subjected to any dedicated morphological identification research or have not appeared in the community-level samples of past assessments. Taxa that appeared both as an OTU and have been identified morphologically in published research are indicated with an asterisk (*).

Group	Class	Order	Family	Genus	SV	SK	CR	Markers	Putative taxon	Reference
Metazoa	*Arachnida	*Trombidiformes	Halacaridae	*Copidognathus*	X	X	X	COI	Trombidiformes	1
				Rhombognathus	X		X	COI	Trombidiformes	1
		Sarcoptiformes	Suidasiidae	*Suidasia*	X		X	COI	-	–
		*Mesostigmata	*Trachytidae	*Uroseius*		X		COI	Trachytidae	1
	Branchiopoda	Diplostraca	Macrotrichidae	*Macrothrix*	X	X	X	COI	–	–
	Chromadorea	Rhabditida	Aphelenchoididae	*Aphelenchoides*		X		COI, 18S	–	–
		Desmodorida	Desmodoridae	*Desmodora*	X	X	X	COI	–	–
		Chromadorida	Chromadoridae	*Dichromadora*	X	X	X	COI	–	–
				Neochromadora	X		X	COI	–	–
	*Clitellata	Crassiclitellata	Lumbricidae	*Cataladrilus*	X	X		COI	–	–
		Tubificida	*Naididae	*Heterodrilus*	X	X		COI	Naididae	1
				Rhyacodrilus	X	X		COI	Naididae	1
				Ainudrilus	X			COI	Naididae	1
	Elardia	Arcellinida	Centropyxidae	*Centropyxis*	X	X	X	COI	–	–
	Enoplea	Enoplida	Oncholaimidae	*Pontonema*			X	COI	–	–
	Eurotatoria	Adinetida	Adinetidae	*Adineta*	X	X	X	COI	–	–
		Ploima	Asplanchnidae	*Asplanchna*		X	X	COI	–	–
		Philodinida	Philodinidae	-		X		COI	–	–
	Flabellinia	Unknown	Vannellidae	*Vannella*	X	X	X	COI	–	–
		Dactylopodida	Paramoebidae	*Cunea*			X	COI	–	–
	Hexanauplia	Harpacticoida	Miraciidae	*Schizopera*	X	X	X	COI	–	–
	*Insecta	Lepidoptera	Gracillariidae	*Phyllonorycter*	X		X	COI	–	–
		*Diptera	*Ceratopogonidae	-	X		X	COI	Ceratopogonidae	1
			*Chironomidae	*Dicrotendipes*	X			COI	*Semiocladius* sp.	1
				Constempellina	X			COI	*Semiocladius* sp.	1
	*Malacostraca	*Decapoda	*Varunidae	**Cyclograpsus*	X			COI	*C. punctatus*	2
		*Amphipoda	*Talitridae	*Orchestoidea*		X		COI	*Euorchestia rectipalma*	1
		*Isopoda	*Idoteidae	*Pentidotea*		X		COI	*Synidotea variegata*	1
		*Tanaidacea	*Tanaididae	**Sinelobus*	X	X	X	COI	*S. stromatoliticus*	3
	*Ostracoda	*Podocopida	Limnocytheridae	*Metacypris*	X	X	X	COI, 18S	*Physocypria capensis*	1
	*Polychaeta	*Sabellida	*Serpulidae	**Ficopomatus*		X		COI	*F. enigmaticus*	4
		*Phyllodocida	*Nereididae	*Perinereis*	X	X	X	COI	*Composetia keiskama*	1
Algae	*Bacillariophyceae	*Naviculales	*Naviculaceae	**Navicula*	X	X	X	rbcL, 18S	*Navicula* sp.	5
				Fistulifera	X	X		rbcl	Naviculaceae	5
			*Diploneidaceae	**Diploneis*	X	X	X	rbcl	*Diploneis* sp.	5
			Amphipleuraceae	*Halamphora*	X		X	rbcl	-	–
			Sellaphoraceae	*Fallacia*			X	rbcl	-	–
		*Bacillariales	Bacillariaceae	**Nitzschia*	X		X	rbcl	*N. scalpelliformis*	5
		*Achnanthales	Achnanthidiaceae	**Achnanthidium*		X		rbcl	*A. minutissimum*	5
				**Planothidium*		X		rbcl	*Planothidium* sp.	5
		*Thalassiophysales	*Catenulaceae	**Amphora*		X		rbcl	*A. coffeiformis*	5
	Coscinodiscophyceae	Chaetocerotales	Chaetocerotaceae	*Chaetoceros*		X		18S	–	–
	Labyrinthulea	Thraustochytrida	Aplanochytriidae	*Aplanochytrium*	X			18S	–	–
	Eustigmatophyceae	Eustigmatales	Neomonodaceae	-		X		rbcL	-	–
	Chrysophyseae	Chromulinales	Ochromonadaceae	*Poterioochromonas*		X		18S	-	–
	Labyrinthulea	Thraustochytrida	Thraustochytriidae	*Thraustochytrium*	X	X		18S	-	–
	Mortierellomycetes	Mortierellales	Mortierellaceae	-			X	18S	-	–
	Tremellomycetes	Trichosporonales	Trichosporonaceae	*Apiotrichum*		X	X	18S	-	–

1: Rishworth et al. (2017b); 2: Rishworth et al. (2017d); 3: Rishworth et al. (2019c).; 4: Miranda et al. (2016); 5: Bornman et al. (2017).

(Coscinodiscophyceae), as well as non-diatom algae of the classes Chrysophyseae, Eustigmatophyceae and Labyrinthulea (Table 2). The 18S sequences were dominated by the OTU of a diatom assigned to the genus *Navicula* (Fig. 3C).

After aggregating taxonomy tables at the family level, the average observed richness was calculated at 11.1, 4.1 and 3.7 for the COI, rbcL and 18S markers, respectively. The Shannon and Simpson diversity indices for COI did not differ between pools, but significant differences were found for rbcL ($P = 0.04$), whereas observed richness was only significant for 18S ($P = 0.04$; Table 3). The Bray-Curtis index of dissimilarity was significantly different between sites for all three markers ($P \leq 0.01$; Fig. 3). PCoA ordination plots for COI and rbcL showed that different replicates from the same pool clustered together (Fig. 3B, D and F). Beta diversity thus indicated that despite some overlap in community composition between sites (Table 2) each site had a characteristic community structure at the family level (Table 3).

The full RDA analysis, as well as axes RDA1 and RDA2, showed statistically significant effects of environmental variables on OTU diversity of microbialite pools (p-values: 0.002, 0.004, and 0.012, respectively). The first two axes explain 40.04 % of the total variance in OTU diversity (Fig. 4). As DIP was collinear with DIN ($r = 0.84$), as well as temperature, salinity and oxygen ($r > 0.98$), only pH and DIP were included as independent variables ($r = 0.58$). Nutrient conditions, represented by DIP, rather than any of the other correlated environmental variables, were chosen because these are known to vary significantly between microbialite pool locations (Rishworth et al., 2017c). The most obvious relationships that constrained OTU diversity were that SV is distinguishable from the other two sites by a stronger positive association with DIP, while constrained OTU diversity at CR was inversely associated with pH.

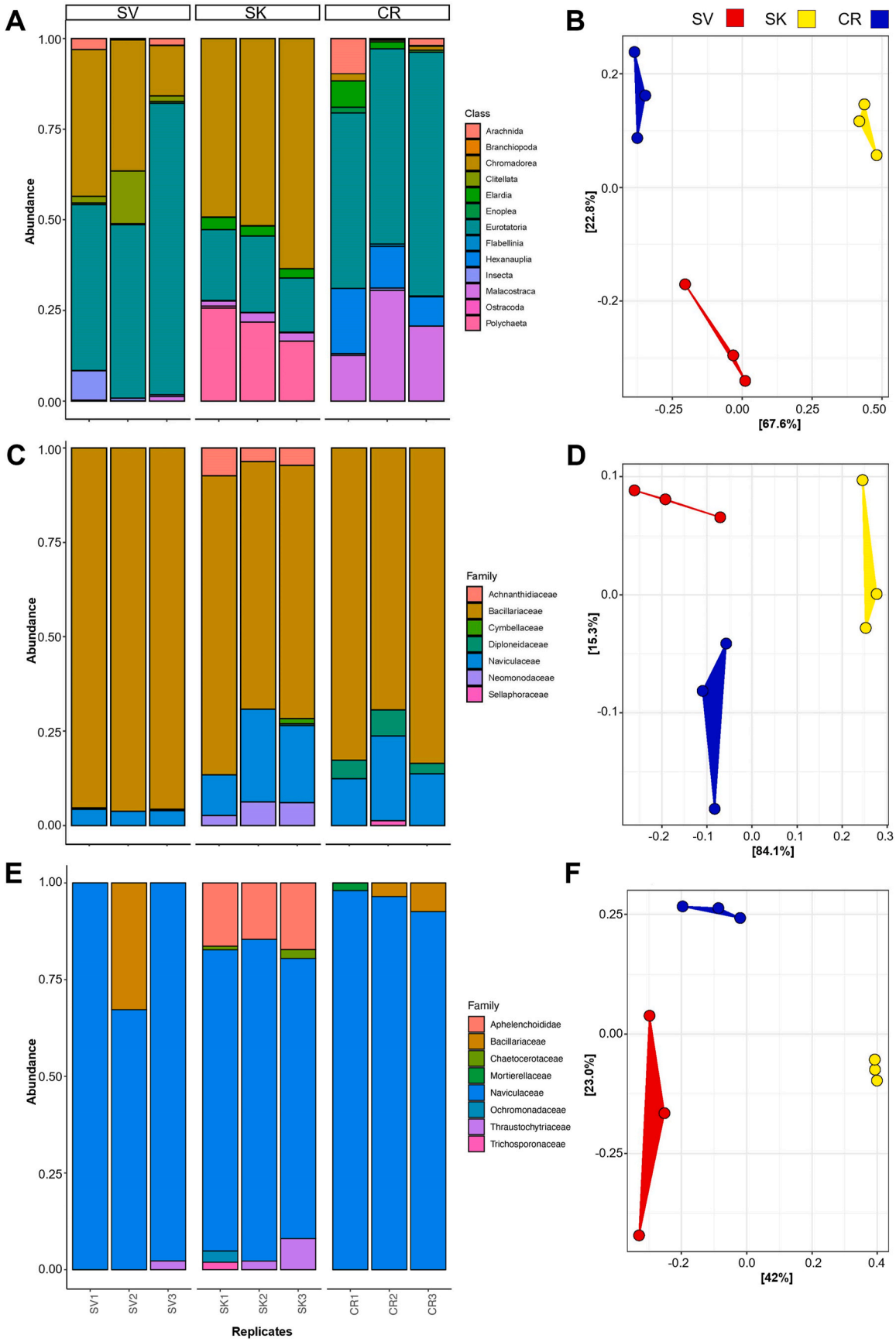

Fig. 3. Bar plots (A, C, and E) indicating relative OTU abundance within classes (COI) and families (rbcL and 18S) for each of three replicate sample per microbialite pool; PCoA ordination plots (B, D and F) based on Bray-Curtis dissimilarity between replicates. **A, B –** COI, **C, D –** rbcL and **E, F –** 18S. SV (Seaview), SK (Schoenmakerskop) and CR (Cape Recife).

Table 3
Alpha and beta diversity for sampling sites. Alpha diversity (averages reported from three replicates) includes observed richness, Chao1 richness, Shannon diversity and Simpson diversity, as well as *P*-values of Kruskal-Wallis tests (comparing alpha diversity indices between sites). Beta diversity was assessed using ANOSIM based on Bray-Curtis dissimilarity. Significant *P*-values are shown in bold. SV – Seaview, SK - Schoenmakerskop, CR - Cape Recife.

COI	SV	SK	CR	*P* (Kruskal Wallis)
Observed	11.6	7.3	11.3	0.08
Shannon	1.18	1.17	1.51	0.20
Simpson	0.51	0.61	0.71	0.11
P (ANOSIM)	**0.002**			
rbcL	SV	SK	CR	*P* (Kruskal Wallis)
Observed	2.67	5.33	4.33	0.10
Shannon	0.22	0.90	0.72	**0.04**
Simpson	0.10	0.46	0.39	**0.04**
P (ANOSIM)	**0.006**			
18S	SV	SK	CR	*P* (Kruskal Wallis)
Observed	3.33	5.33	2.33	**0.04**
Shannon	0.34	0.70	0.19	0.11
Simpson	0.19	0.34	0.09	0.25
P (ANOSIM)	**0.01**			

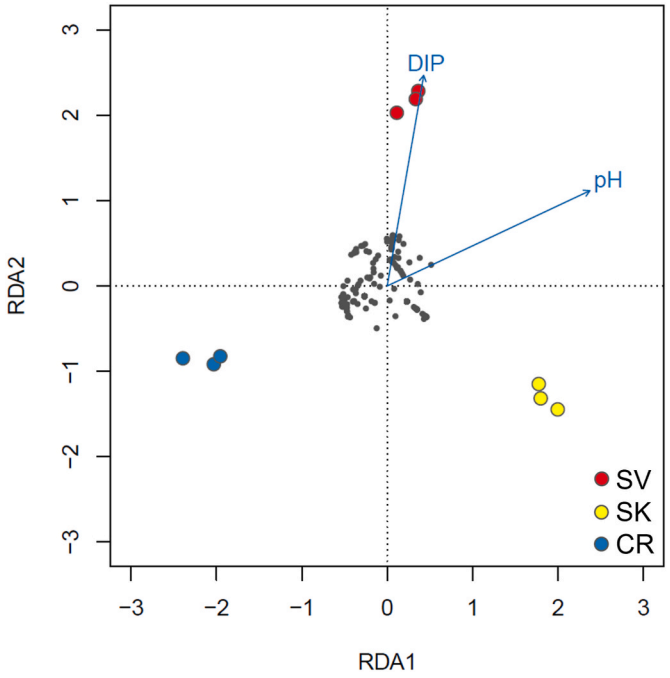

Fig. 4. Redundancy analysis (RDA) of OTU diversity across three markers (COI, rbcL and 18S) in relation to standardised, non-collinear environmental variables (dissolved inorganic phosphorus: DIP, and pH) per microbialite pool: SV (Seaview), SK (Schoenmakerskop) and CR (Cape Recife). Grey dots represent OTUs assessed.

4. Discussion

This is the first study to use a multi-marker eDNA metabarcoding approach in assessing the community composition and diversity of eukaryotes in South African supratidal spring-fed microbialite pools.

4.1. Metazoan diversity

Operational taxonomic units (OTUs, which represent putative species or families depending on the markers used) assigned to the metazoans based on COI sequences were mostly identified as peracarid crustaceans, rotifers and nematodes, with other taxa being less common. While the diversity of major metazoan families, such as larger peracarid crustaceans and polychaete worms, in microbialite pools is well documented since these taxa have only a few representatives in these habitats, very little is known about the smaller meiofauna. Metazoans are known to disrupt microbialite layering, and a conventional hypothesis is that modern stromatolites are found in areas where metazoan activity is scarce (Rishworth et al., 2016b). However, there are some locations in the Bahamas (Garrett, 1970), Mexico (Garcia-Pichel et al., 2004; Dinger et al., 2006), Canada (Bonacolta et al., 2024) and Australia (Konishi et al., 2001; Allen et al., 2009; Edgcomb et al., 2014) where metazoans and stromatolites coexist (Tarhan et al., 2013). Similarly, the metazoans living in microbialites along the South African coast do not completely inhibit the formations by their burrowing or grazing behaviours. Earlier studies on larger macrofauna indicate that they consume the abundance of macroalgae found within these pools rather than feeding on the microbialite material itself (Rishworth et al., 2017d, 2018; Hawkes et al., 2024).

Several OTUs associated with meiofauna were found at all three sites, which suggests that they may represent core components of the metazoan community, and some may even be endemic to these systems. These include the branchiopod crustacean identified as *Macrothrix* sp., the chromadorean nematode worms identified as *Desmodora* sp. and *Dichromadora* sp., the amoebozoans identified as *Centropysix* sp. and *Vannella* sp., the rotifer identified as *Adineta* sp., and the copepod identified as *Schizophera* sp. The genus-level identifications reported here need to be interpreted with caution since it is possible that some OTUs not only represent species that are still missing from public DNA barcoding reference databases, but they may even represent putative new species that have never been scientifically described. As the metazoans associated with microbialite pools comprise comparatively few macrofauna species (Rishworth et al., 2016b, 2017b, 2020) efforts aimed at describing the core meiofauna of these unique coastal habitats represents a realistic and important aim for the near future. To investigate the meiofauna in more detail, we recommend including 18S V4 (with primers Uni18S and Uni18SR: Zhan et al., 2013) as an additional marker in the multi-marker approach, as it detects far more meiofauna taxa such as Platyhelminthes, Nematoda, Gastrotricha, Xenacoelomorpha and Entoprocta compared to COI (Ohnesorge et al., 2023).

Earlier studies on other microbialites indicate that meiofauna is an important functional component of these habitats. Protists such as foraminifera might facilitate the transition of laminated stromatolites to clotted thrombolites (Bernhard et al., 2023). Metazoan meiofauna alter the structure of microbialites through grazing and bioturbation pressures and affect microbialite formation processes through production of exopolymeric substances (see review in Bonacolta et al., 2024). Nematodes in the Great Salt Lake microbialites occur in high abundance in these biofilms and have a broad salinity tolerance with a unique identity (Jung et al., 2024). Bonacolta et al. (2024), expanding on the work of Edgcomb et al. (2014), reported that the overall composition of the protist community within microbialites is responsive to salinity drivers and comprises a core assemblage that overlaps between microbialite sites in terms of functional groups. The meiofauna associated with most microbialites is likely diverse. For example, in the Bacalar Lake in Mexico, at least 22 nematode genera were identified, comprising all functional feeding groups characteristic of nematodes (de Jesús-Navarrete et al., 2021). In contrast, only four genera of nematodes were observed as COI OTUs in this study. None of these can be conclusively linked to putative species because no dedicated research has been

conducted on nematodes despite these having been regularly observed within the microbialite matrix during incidental macrofauna sample processing (GMR pers. obs.). Expanding the types of markers used to include 18S V4, as highlighted above, would be important in this regard to capture the full scope of occurrence, diversity and role of meiofauna such as nematodes in these ecosystems.

The COI database further comprised insects and arachnids. Chironomid larvae are important macroinvertebrates that are common in brackish water systems, including estuaries (Teske and Wooldridge, 2001) and microbialite pools (Rishworth et al., 2017b), and OTUs associated with two genera (*Pontomyia* and *Constempellina*) were identified here. The association of terrestrial arachnids and insects (e.g. mites and moths) with the pools is less clear. Some may be present in the freshwater springs feeding the microbialite pools during their larval stages, and their eDNA was carried into the brackish water section that was sampled. Aquatic mites have been regularly observed in the microbialite pools and likely are a non-incidental component of the macrofauna community (Rishworth et al., 2017b; Weston et al., 2018). Other arthropod OTUs may be representatives of the terrestrial fauna that occurs in the vicinity of the pools, and their eDNA was collected as an allochthonous input. The presence of unusual taxa such as the first regional record of a halophilic centipede, a group that is typically terrestrial, have already been documented from the microbialite pools (*Tuoba* cf. *poseidonis*: Rishworth et al., 2016b).

4.2. Algal diversity

Metabarcoding results of rbcL and 18S indicate the presence of a few core algae in all three pools. Some diatom OTUs were not consistent between both markers, highlighting the value of a multi-marker approach. The diatom genera *Navicula* (both markers) and *Nitzschia* (rbcL only) were ubiquitously identified, which points to the critical role that some algae may play as ecosystem-engineers within the biofilm formations of South African microbialites (Bornman et al., 2017). However, the exact role of the different algal species is not clear and represents an area of ongoing research. It has been suggested that diatoms, together with cyanobacteria, are the phototrophs responsible for microbialite growth and accretion (Stolz, 2003; Rishworth et al., 2016a). A similar functional role has been suggested for diatoms in microbialites found in Australia, Brazil, the Bahamas, and in South Africa (John et al., 2009; Edgcomb et al., 2014; Casaburi et al., 2016; Bornman et al., 2017; Laut et al., 2019; Rishworth et al., 2016a, 2020; Bonacolta et al., 2024). Less abundant diatoms identified from the South African pools, such as *Amphora* sp. and *Achnanthidium* sp., have been reported from microbialite formations around the world, as well as in hypersaline lakes in northwestern Argentina (Farías et al., 2011).

4.3. Taxonomic assignments

More broadly for all eukaryote taxa, there were clear systemic differences in the taxonomic rank assignments between the metabarcoding approach used here (Fig. 5) compared to prior traditional taxonomic assessments (Miranda et al., 2016; Bornman et al., 2017; Rishworth et al., 2017b, 2017d, Rishworth et al., 2019c, 2020). At the class level, traditional methods have missed just under half of the classes that the metabarcoding approach identified, most of these being linked to the meiofauna (Fig. 5). However, the metabarcoding approach recognised 75 % of the classes that traditional methods have identified. Across all levels of taxonomy, between 19 and 38 % of the OTUs identified have not been identified using traditional methods. Similarly, at the family and genus levels, traditional methods have identified at least 52–56 % of the 93–103 families and genera recorded from spring-fed supratidal microbialite pools that have been picked up using metabarcoding. A paltry 10 % of OTUs could be assigned putative genera (Table 3; Fig. 5). This points to the gaps in local reference libraries (*sensu* Singh et al., 2021), but also the clear benefit in a multi-method integrated taxonomic approach to community-level research on microbialites to encompass the full diversity of the ecosystem.

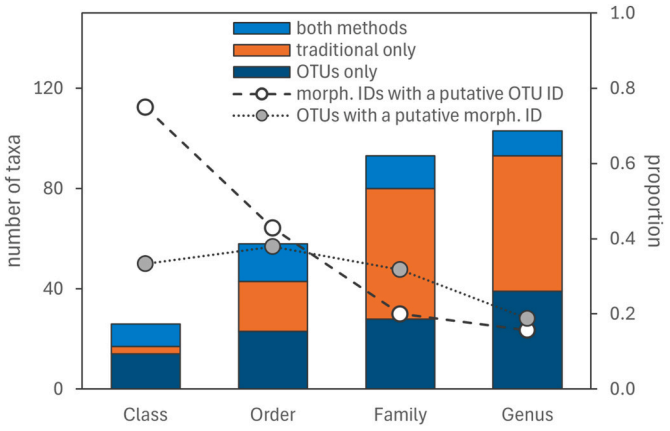

Fig. 5. Number of eukaryote taxa (excluding vertebrates) identified across the three microbialite sites (SV, SK, CR) using metabarcoding (this study) compared to all eukaryotes identified previously (as published in: Miranda et al., 2016; Bornman et al., 2017; Rishworth et al., 2017b, 2017d, Rishworth et al., 2019c, 2020). Taxa are grouped according to increasing resolution (class, order, family and genus level). Coloured bars indicate the number of taxa identified consistently by both methods (light blue), those identified using traditional methods only (orange) and those identified using metabarcoding methods only in this study (dark blue). The proportion of taxa identified using traditional methods which are also observed in this study as OTUs (white circles) and the proportion of taxa observed as OTUs in this study which have also been identified traditionally (grey circles) are also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

A comparison with morphological studies shows that several metazoan taxa that are known to consistently occur in the microbialite pools (Rishworth et al., 2017b, 2017d; Weston et al., 2018) could not be conclusively identified to genus or species level, as they are not yet represented in the DNA reference databases. Others may not have been successfully amplified or sequenced, and therefore collapsed to a broader taxon (e.g. the isopod *Cyathura estuaria*, the amphipods *Melita zeylanica*, *Euorchestia rectipalma*, and *Americorophium triaenonyx,* and the shrimp *Palaemon peringueyii*, all of which are prominent features of these microbialite pools: Rishworth et al., 2017b, 2017b, 2020). The rbcL and 18S marker amplified very different taxa, and for each, a different diatom taxon was identified as being dominant (*Nitzschia* for rbcL, *Navicula* for 18S). This suggests that documenting the microbialite-associated eukaryote community (including meiofauna and zooplankton) will also require a multi-marker approach that may require additional markers (e.g., 18S V4) or more taxon-specific DNA primers.

Overall, the metabarcoding approach shows that the metazoan community within stromatolite pools is likely to be more extensive and diverse than what traditional approaches alone have observed. It is important to note that no published inventory based on morphological studies exists for the zooplankton or meiofauna of South African microbialite pools, making it difficult to directly compare the biodiversity data obtained in this study. This absence highlights the significant potential of multi-species DNA barcoding methods similar to that employed here for conducting comparative biodiversity analyses across various components of ecosystems. At the same time, it points to the critical need for the expansion of local reference sequence databases for a more accurate taxonomic rank assignment.

4.4. Spatial patterns

While some core taxa were found ubiquitously (Table 2), the composition and distribution of communities varied significantly

between sites (Table 3). This aligns with previous work at the microbial and macrofaunal level which documented clear site-specific divergence (Rishworth et al., 2017b, 2020; Neuhaus et al., 2024). It suggests that a baseline fauna and flora play a critical role in the formation and function of microbialite-associated ecosystems, and other taxa are opportunistic inhabitants establishing themselves occasionally and possibly temporarily in microbialite pools. Evidence for this is seen in some species that recruit to these environments during specific periods of their life-history (Rishworth et al., 2017a; Grundlingh et al., 2023) as opportunistic habitat occupants (Miranda et al., 2016; Rishworth et al., 2024). This suggests that the microbialite biofilms and pools function as foundational habitats for some taxa.

The reason for the spatial variation in the eukaryote community structure remains unclear, although there is some evidence that environmental variability between sites is important. An earlier study reports a link between abiotic and biotic factors, and the abundance of metazoans within the pools (Rishworth et al., 2017b). For example, there is a gradient of nutrient enrichment within the groundwater entering the pools from CR (most pristine) to SV (most eutrophic) that might drive these spatial patterns (Rishworth et al., 2017c). The RDA analysis supports this observation across the eukaryote community, suggesting that SV, as the most nutrient-enriched site, is distinguishable from the other two sites by having higher levels of DIP (Fig. 4). Mechanistic links for this association are beyond the scope of this study, but prior research has demonstrated the important association between nutrients and microbialite communities (Forbes et al., 2010; Rishworth et al., 2017c; Doddy et al., 2019). Additionally, the intrinsic inter-site differences in macrostructure of the three pools sampled in this study (Edwards et al., 2017), and the consequent niche variability (Weston et al., 2018, 2019) might contribute to the observed differences between sites. For example, this has previously been observed for the isopod *Cyathura estuaria*, which has only been documented at the CR microbialite pools, but not at SV or SK (Rishworth et al., 2017b).

The heterogeneity in the distribution of identified taxa could also arise as a result of sequencing non-target marine, estuarine or terrestrial species that do not reside permanently within the microbialite pools, but there are also indications that the metabarcoding at the selected sequencing depth may have failed to detect some resident key species that are known to be associated with specific areas where microbialite pools are present. For example, chironomid larvae were only found at SV in the present study using metabarcoding techniques, but they have also been reported from SK and CR (Rishworth et al., 2017b).

5. Conclusion

This study demonstrates the potential of a multi-marker metabarcoding approach as a tool to assess the community composition and diversity of eukaryotes in South African supratidal spring-fed microbialite pools. However, the results of eDNA metabarcoding studies that are conducted in underrepresented geographical areas, or for taxa without reference barcoding sequences, have limitations that need to be considered, as portrayed in Fig. 5 (see also Singh et al., 2021). A comparison with previous work done using traditional taxonomic approaches shows that metabarcoding of biodiversity did not capture the full range of organisms known to occur in these South African microbialite pools. Reasons for this include a lack of sequencing convergence of OTUs given the restricted effort spent elsewhere in terms of cataloguing and barcoding appropriate marker regions of local taxa, limited effort having been spent cataloguing multiple markers of single organisms or species, and an incomplete understanding of the utility of additional markers that might be better suited to the identification of some species or assemblages. Future efforts therefore need to prioritise addressing these uncertainties before the full potential use of eDNA amplification as a community biomonitoring and assessment tool can be fully realised. This is important as a better understanding of the biodiversity within these unique coastal ecosystems is vital to understanding their role in facilitating connectivity between other coastal ecosystems in southern Africa, and to identify additional species that may be endemic to the region's microbialite pools. Our research highlights the value of an integrative approach to taxonomy in achieving this.

CRediT authorship contribution statement

Arsalan Emami-Khoyi: Writing – original draft, Project administration, Methodology, Formal analysis, Conceptualization. **Claudia M. Schnelle:** Writing – original draft, Visualization, Methodology, Formal analysis. **Dave R. Clark:** Writing – review & editing. **Silke Laakmann:** Writing – review & editing, Conceptualization. **Peter R. Teske:** Writing – review & editing, Supervision. **Gavin M. Rishworth:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Conceptualization.

Data availability

All data used to produce the current manuscript are reproduced in the supplementary material or will be made available upon request. Sequences generated from this project have been submitted to the National Centre for Biotechnology Information (NCBI) under BioProject ID PRJNA1187521 https://submit.ncbi.nlm.nih.gov/subs/sra/SUB14873335/overview.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was primarily funded by a grant provided to GMR by the National Research Foundation (NRF) of South Africa (UID: 129402). It was also supported by the DSI/NRF South African Research Chairs Initiative (Shallow Water Ecosystems; UID: 84375) and the DSI/NRF Community of Practice (Algoa Bay Marine Spatial Planning; UID: 110612). Grants provided by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG; UID: MA 3274/16-1) and the United Kingdom's Research and Innovation Natural Environmental Research Council (NERC; UID: NE/V00834X/1) supported the collaboration with SL and DC, respectively. SL was financially supported through the POF IV, subtopic 6.1 research program of the HIFMB as part of the Helmholtz Association of German Research Centres. Carla Dodd is thanked for producing the study site map. This research is an output of the Supratidal Spring-fed Living Microbialite Ecosystems (SSLiME) Project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2025.109284.

Data availability

Data are available in the Supplementary Material or the online NCBI database, as outlined in the manuscript.

References

Alberdi, A., Aizpurua, O., Gilbert, M.T.P., Bohmann, K., 2018. Scrutinizing key steps for reliable metabarcoding of environmental samples. Methods Ecol. Evol. 9, 134–147. https://doi.org/10.1111/2041-210X.12849.

Allen, M.A., Goh, F., Burns, B.P., Neilan, B.A., 2009. Bacterial, archaeal and eukaryotic diversity of smooth and pustular microbial mat communities in the hypersaline

lagoon of Shark Bay. Geobiology 7, 82–96. https://doi.org/10.1111/j.1472-4669.2008.00187.x.
Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.
Andrews, S., 2010. FastQC: a Quality Control Tool for High Throughput Sequence Data.
Bernhard, J.M., Fisher, L.A., Murphy, Q., Sen, L., Yeh, H.D., Louyakis, A., Gomaa, F., Reilly, M., Batta-Lona, P.G., Bucklin, A., 2023. Transition from stromatolite to thrombolite fabric: potential role for reticulopodial protists in lake microbialites of a Proterozoic ecosystem analog. Front. Microbiol. 14, 1210781. https://doi.org/10.3389/fmicb.2023.1210781.
Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.
Bonacolta, A.M., Visscher, P.T., Del Campo, J., White III, R.A., 2024. The eukaryome of modern microbialites reveals distinct colonization across aquatic ecosystems. npj Biofilms Microbiomes 10, 78. https://doi.org/10.1038/s41522-024-00547-z.
Bornman, T.G., Perissinotto, R., Dąbek, P., Rishworth, G., 2017. The diatoms of shore platform extant stromatolite ecosystems in South Africa. Phycologia 56, 23. https://doi.org/10.5194/bg-15-2189-2018.
Bray, J.R., Curtis, J.T., 1957. An ordination of the upload forest communities of southern Wisconsin. Ecol. Monogr. 27, 325–349. https://doi.org/10.2307/1942268.
Casaburi, G., Duscher, A.A., Reid, R.P., Foster, J.S., 2016. Characterization of the stromatolite microbiome from Little Darby Island, The Bahamas using predictive and whole shotgun metagenomic analysis. Environ. Microbiol. 18, 1452–1469. https://doi.org/10.1111/1462-2920.13094.
Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18, 117–143. https://doi.org/10.1111/j.1442-9993.1993.tb00438.x.
Colwell, R.K., 2009. Biodiversity: concepts, patterns, and measurement. The Princeton guide to ecology 663, 257–263. https://doi.org/10.1515/9781400833023.257.
Cooper, A., Smith, A., Rishworth, G., Dodd, C., Forbes, M., Cawthra, H., Anderson, C., 2022. Microbialites of modern siliciclastic rock coasts. J. Sediment. Res. 92, 619–634. https://doi.org/10.2110/jsr.2021.071.
de Jesús-Navarrete, A., Yanez-Montalvo, A., Falcón, L.I., Vargas-Espósitos, A., 2021. Nematode fauna associated with freshwater microbialites in Bacalar Lake, Quintana Roo, Mexico. Limnology 22, 347–355. https://doi.org/10.1007/s10201-021-00662-2.
Dinger, E.C., Hendrickson, A., Winsborough, B.M., Marks, J.C., 2006. Role of fish in structuring invertebrates on stromatolites in Cuarto Ciénegas, México. Hydrobiologia 563, 407–420. https://doi.org/10.1007/s10750-006-0040-4.
Dodd, C., Anderson, C.R., Rishworth, G.M., Perissinotto, R., van Niekerk, X., 2021. Metazoan activity facilitates passive sediment trapping in modern supratidal microbialites: revealed using μ-CT-scanning and microscopy. Geobiology 19, 585–600. https://doi.org/10.1111/gbi.12456.
Doddy, P., Roden, C.M., Gammell, M.P., 2019. Nutrient pollution degrades microbialites in Lough Carra, an Irish marl lake. Aquat. Microb. Ecol. 83, 203–209. https://doi.org/10.3354/ame01917.
du Toit, H.D., Rishworth, G.M., Strydom, N.A., Welman, S., 2024. High levels of metacercarial infestation (Family: Diplostomidae) do not affect host energetics and swimming performance in the Epaulette goby (*Coryogalops sordidus*, Gobiidae). J. Fish. Biol. 104, 1165–1179. https://doi.org/10.1111/jfb.15657.
Edgar, R.C., Flyvbjerg, H., 2015. Error filtering, pair assembly and error correction for next-generation sequencing reads. Bioinformatics 31, 3476–3482. https://doi.org/10.1093/bioinformatics/btv401.
Edgcomb, V.P., Bernhard, M., Summons, E., Orsi, W., Beaudoin, D., Visscher, P.T., 2014. Active eukaryotes in microbialites from highborne cay, Bahamas, and hamelin pool (shark Bay), Australia. ISME J. 8, 418–429. https://doi.org/10.1038/ismej.2013.130.
Edwards, M.J.K., Anderson, C.R., Perissinotto, R., Rishworth, G.M., 2017. Macro-and meso-fabric structures of peritidal tufa stromatolites along the Eastern Cape coast of South Africa. Sediment. Geol. 359, 62–75. https://doi.org/10.1016/j.sedgeo.2017.08.006.
Farías, M.E., Poiré, D.G., Arrouy, M.J., Albarracin, V.H., 2011. Modern stromatolite ecosystems at alkaline and hypersaline high-altitude lakes in the Argentinean Puna. Stromatolites: interaction of microbes with sediments 427–441. https://doi.org/10.1007/978-94-007-0397-1_19.
Forbes, M., Vogwill, R., Onton, K., 2010. A characterisation of the coastal tufa deposits of south–west Western Australia. Sediment. Geol. 232, 52–65. https://doi.org/10.1016/j.sedgeo.2010.09.009.
Garcia-Pichel, F., Al-Horani, F.A., Farmer, J.D., Ludwig, R., Wade, B.D., 2004. Balance between microbial calcification and metazoan bioerosion in modern stromatolitic oncolites. Geobiology 2, 49–57. https://doi.org/10.1111/j.1472-4669.2004.00017.x.
Garner, T.W., Cooper, J.A.G., Smith, A.M., Rishworth, G.M., Forbes, M., 2024. Reviews and syntheses: tufa microbialites on rocky coasts – towards an integrated terminology. Biogeosciences 21, 4785–4807. https://doi.org/10.5194/bg-21-4785-2024.
Garrett, P., 1970. Phanerozoic stromatolites: noncompetitive ecologic restriction by grazing and burrowing animals. Science 169, 171–173. https://doi.org/10.1126/science.169.3941.171.
Geller, J., Meyer, C., Parker, M., Hawk, H., 2013. Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. Mol. Ecol. Res. 13, 851–861. https://doi.org/10.1111/1755-0998.12138.
Giebner, H., Langen, K., Bourlat, S.J., Kukowka, S., Mayer, C., Astrin, J.J., Misof, B., Fonseca, V.G., 2020. Comparing diversity levels in environmental samples: DNA sequence capture and metabarcoding approaches using 18S and COI genes. Mol. Ecol. Res. 20, 1333–1345. https://doi.org/10.1111/1755-0998.13201.
Grundlingh, P.L., Rishworth, G.M., Strydom, N.A., 2023. Could extremophilia be a life history strategy in late-stage larvae of *Sparodon durbanensis* (Family Sparidae)? Estuar. Coast Shelf Sci. 294, 108505. https://doi.org/10.1016/j.ecss.2023.108505.
Harris, L., Adams, J., Dayaram, A., Dunga, L., Job, N., Kirkman, S., Lamberth, S., Pfaff, M., Raw, J., Rishworth, G.M., Robbins, A., Sink, K., Skowno, A., van Deventer, H., van Niekerk, L.V., 2025. Cross-realm biodiversity profile of the South African coastal zone. Afr. J. Mar. Sci. https://doi.org/10.2989/1814232X.2025.2463342.
Harrison, J.B., Sunday, J.M., Rogers, S.M., 2019. Predicting the fate of eDNA in the environment and implications for studying biodiversity. Proc. R. Soc. B 286, 20191409. https://doi.org/10.1098/rspb.2019.1409.
Hawkes, S.A., Welman, S., Human, L.R., Lemley, D.A., Smit, L., Steyn, P., Rishworth, G. M., 2024. Nutritional qualities partially predict diet of amphipod grazers: implications for modern coastal biofilms in microbialite pools. Mar. Ecol. Prog. Ser. 736, 19–33. https://doi.org/10.3354/meps14585.
John, J., Hay, M., Paton, J., 2009. Cyanobacteria in benthic microbial communities in coastal salt lakes in Western Australia. Algal. Stud. 130, 125. https://doi.org/10.1127/1864-1318/2009/0130-0125.
Jung, J., Loschko, T., Reich, S., Rassoul-Agha, M., Werner, M.S., 2024. Newly identified nematodes from the Great Salt Lake are associated with microbialites and specially adapted to hypersaline conditions. Proc. R. Soc. B 291, 20232653. https://doi.org/10.1098/rspb.2023.2653.
Kahlke, T., Ralph, P.J., 2019. Basta – taxonomic classification of sequences and sequence bins using last common ancestor estimations. Methods Ecol. Evol. 10, 100–103. https://doi.org/10.1111/2041-210X.13095.
Kans, J., 2024. Entrez direct: E-utilities on the UNIX command line. In: Anonymous Entrez Programming Utilities Help. National Center for Biotechnology Information (US) [Internet].
Konishi, Y., Prince, J., Knott, B., 2001. The fauna of thrombolitic microbialites, Lake Clifton, Western Australia. Hydrobiologia 457, 39–47. https://doi.org/10.1023/A:1012229412462.
Kruskal, W.H., Wallis, W.A., 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc. 47, 583–621. https://doi.org/10.2307/2280779.
Kwon, S., Park, S., Lee, B., Yoon, S., 2013. In-depth analysis of interrelation between quality scores and real errors in illumina reads. In: 2013 35th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC). IEEE, pp. 635–638.
Laut, L., Figueiredo, M.S., Lorini, M.L., Belart, P., Clemente, I., Martins, M.V.A., Mendonca Filho, J.G., Laut, V., 2019. Diatoms from the most hypersaline lagoon in Brazil: Vermelha Lagoon. Cont. Shelf Res. 181, 111–123. https://doi.org/10.1016/j.csr.2019.05.001.
Leray, M., Yang, J.Y., Meyer, C.P., Mills, S.C., Agudelo, N., Ranwez, V., Boehm, J.T., Machida, R.J., 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Front. Zool. 10, 1–14. https://doi.org/10.1186/1742-9994-10-34.
Liu, M., Zhao, Y., Sun, Y., Li, Y., Wu, P., Zhou, S., Ren, L., 2020. Comparative study on diatom morphology and molecular identification in drowning cases. Forensic Sci. Int. 317, 110552. https://doi.org/10.1016/j.forsciint.2020.110552.
Liu, M., Zhao, Y., Sun, Y., Wu, P., Zhou, S., Ren, L., 2024. Diatom DNA barcodes for forensic discrimination of drowning incidents. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 367. https://doi.org/10.1093/femsle/fnaa145.
Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17, 10–12. https://doi.org/10.14806/ej.17.1.200.
McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLOS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217.
Miranda, N.A., Kupriyanova, E.K., Rishworth, G.M., Peer, N., Bornman, T.G., Bird, M.S., Perissinotto, R., 2016. An invasive polychaete species found in living marine stromatolites. Aquat. Invasions 11, 257–266. https://doi.org/10.3391/ai.2016.11.3.04.
Neuhaus, G.F., Aron, A.T., Isemonger, E.W., Petras, D., Waterworth, S.C., Madonsela, L. S., Gentry, E.C., Siwe Noundou, X., Kalinski, J.J., Polyzois, A., 2024. Environmental metabolomics characterization of modern stromatolites and annotation of ibhayipeptolides. PLOS One 19, e0303273. https://doi.org/10.1371/journal.pone.0303273.
Ohnesorge, A., John, U., Taudien, S., Neuhaus, S., Kuczynski, L., Laakmann, S., 2023. Capturing drifting species and molecules—lessons learned from integrated approaches to assess marine metazoan diversity in highly dynamic waters. Environmental DNA 5, 1541–1556. https://doi.org/10.1002/edn3.478.
Oksanen, J., et al., 2024. Vegan: community ecology package. R package version 2.6-6.1 2, 1–295. https://doi.org/10.32614/CRAN.package.vegan.
Perissinotto, R., Bornman, T.G., Steyn, P., Miranda, N.A.F., Dorrington, R.A., Matcher, G. F., Strydom, N., Peer, N., 2014. Tufa stromatolite ecosystems on the South African south coast. South Afr. J. Sci. 110, 1–8. https://doi.org/10.1590/sajs.2014/20140011.
R Core Team, 2024. R: a Language and Environment for Statistical Computing.
Reid, R.P., Visscher, P.T., Decho, A.W., Stolz, J.F., DesMarais, D.J., 2000. The role of microbes in accretion, lamination and early lithification of modern marine stromatolites. Nature 406, 989–992. https://doi.org/10.1038/35023158.

Riding, R., 2010. The nature of stromatolites: 3,500 million years of history and a century of research. In: Reitner, J., Queric, N., Arp, G. (Eds.), Advances in Stromatolite Geobiology. Springer-Verlag, Berlin, pp. 29–76.

Rishworth, G.M., et al., 2019a. Box 3. South African Peritidal Stromatolites. South African National Biodiversity Assessment 2018. Technical Report Volume 5: Coast.

Rishworth, G.M., Strydom, N.A., Perissinotto, R., 2017a. Fishes associated with living stromatolite communities in peritidal pools: predators, recruits and ecological traps. Mar. Ecol. Prog. Ser. 153–167. https://doi.org/10.3354/meps12300.

Rishworth, G.M., Edwards, M.J., Cónsole-Gonella, C., Perissinotto, R., 2019b. Modern active microbialite-metazoan relationships in peritidal systems on the Eastern Cape coast of South Africa: ecological significance and implication for the palaeontological record. J. Afr. Earth Sci. 153, 1–8. https://doi.org/10.1016/j.jafrearsci.2019.02.013.

Rishworth, G.M., Perissinotto, R., Bird, M.S., 2017b. Patterns and drivers of benthic macrofaunal communities dwelling within extant peritidal stromatolites. Limnol. Oceanogr. 62. https://doi.org/10.1002/lno.10563.

Rishworth, G.M., Perissinotto, R., Bird, M.S., Pelletier, N., 2018. Grazer responses to variable macroalgal resource conditions facilitate habitat structuring. R. Soc. Open Sci. 5, 2227–2242. https://doi.org/10.1098/rsos.171428, 171428.

Rishworth, G.M., Perissinotto, R., Bornman, T.G., Lemley, D.A., 2017c. Peritidal stromatolites at the convergence of groundwater seepage and marine incursion: patterns of salinity, temperature and nutrient variability. J. Mar. Syst. 167, 68–77. https://doi.org/10.1016/j.jmarsys.2016.11.010.

Rishworth, G.M., van Elden, S., Perissinotto, R., Miranda, N.A., Steyn, P., Bornman, T.G., 2016a. Environmental influences on living marine stromatolites: insights from benthic microalgal communities. Environ. Microbiol. 18, 503–513. https://doi.org/10.1111/1462-2920.13116.

Rishworth, G.M., Wooldridge, T.H., Bakken, T., Miranda, N.A.F., Perissinotto, R., Simon, C.A., 2024. Niche appropriation by a widespread nereidid polychaete within estuarine-like modern coastal stromatolite pools. Estuar. Coast Shelf Sci. 300. https://doi.org/10.1016/j.ecss.2024.108708.

Rishworth, G.M., Perissinotto, R., Błażewicz, M., 2019c. *Sinelobus stromatoliticus* sp. nov. (Peracarida: Tanaidacea) found within extant peritidal stromatolites. Mar. Biodivers. 49, 783–794. https://doi.org/10.1007/s12526-018-0851-3.

Rishworth, G.M., Dodd, C., Perissinotto, R., Bornman, T.G., Adams, J.B., Anderson, C.R., Cawthra, H.C., Dorrington, R.A., Du Toit, H., Edworthy, C., Gibb, R.A., Human, L.R. D., Isemonger, E.W., Lemley, D.A., Miranda, N.A.F., Peer, N., Raw, J.L., Smith, A.M., Steyn, P., Strydom, N.A., Teske, P.R., Welman, S., 2020. Modern supratidal microbialites fed by groundwater: functional drivers, value and trajectories. Earth Sci. Rev. 210, 103364. https://doi.org/10.1016/j.earscirev.2020.103364.

Rishworth, G.M., Perissinotto, R., Bird, M.S., 2016b. Coexisting living stromatolites and infaunal metazoans. Oecologia 182, 539–545. https://doi.org/10.1007/s00442-016-3683-5.

Rishworth, G.M., Perissinotto, R., Bird, M.S., Strydom, N.A., Peer, N., Miranda, N.A.F., Raw, J.L., 2017d. Non-reliance of metazoans on stromatolite-forming microbial mats as a food source. Sci. Rep. 4, 42614. https://doi.org/10.1038/srep42614.

Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4, e2584. https://doi.org/10.7717/peerj.2584.

Shannon, C.E., 1948. A mathematical theory of communication. BSTJ 27, 379–423. https://doi.org/10.1002/j.1538-7305.1948.tb01338.x.

Simpson, E.H., 1949. Measurement of diversity. Nature 163, 688. https://doi.org/10.1038/163688a0.

Singh, S.P., Groeneveld, J.C., Huggett, J., Naidoo, D., Cedras, R., Willows-Munro, S., 2021. Metabarcoding of marine zooplankton in South Africa. Afr. J. Mar. Sci. 43, 1–13. https://doi.org/10.2989/1814232X.2021.1919759.

Smith, A.M., Andrews, J.E., Uken, R., Thackeray, Z., Perissinotto, R., Leuci, R., Marcabell, A., 2011. Rock pool tufa stromatolites on a modern South African wave-cut platform: partial analogues for Archaean stromatolites? Terra Nova 23, 375–381. https://doi.org/10.1111/j.1365-3121.2011.01022.x.

Smith, A.M., Uken, R., 2003. Living marine stromatolites at Kei River mouth: news and views. South Afr. J. Sci. 99, 200. https://hdl.handle.net/10520/EJC97655.

Stolz, J.F., 2003. Structure of marine biofilms: flat laminated mats and modern marine stromatolites. In: Anonymous Fossil and Recent Biofilms: A Natural History of Life on Earth. Springer, pp. 65–76.

Suarez-Gonzalez, P., Benito, M.I., Quijada, I.E., Mas, R., Campos-Soto, S., 2019. 'Trapping and binding': a review of the factors controlling development of fossil agglutinated microbialites and their distribution in space and time. Earth Sci. Rev. 194, 184–215. https://doi.org/10.1016/j.earscirev.2019.05.007.

Taberlet, P., Bonin, A., Zinger, L., Coissac, E., 2018. Environmental DNA: for Biodiversity Research and Monitoring. Oxford University Press.

Tarhan, L.G., Planavsky, N.J., Laumer, C.E., Stolz, J.F., Reid, R.P., 2013. Microbial mat controls on infaunal abundance and diversity in modern marine microbialites. Geobiology 11, 485–497. https://doi.org/10.1111/gbi.12049.

Teske, P.R., Wooldridge, T.H., 2001. A comparison of the macrobenthic faunas of permanently open and temporarily open/closed South African estuaries. Hydrobiologia 464, 227–243. https://doi.org/10.1023/A:1013995302300.

Vasar, M., Davison, J., Moora, M., Sepp, S., Anslan, S., Al-Quraishy, S., Bahram, M., Bueno, C.G., Cantero, J.J., Fabiano, E.C., 2023. Metabarcoding of soil environmental DNA to estimate plant diversity globally. Front. Plant Sci. 14, 1106617. https://doi.org/10.3389/fpls.2023.1106617.

Waterworth, S.C., Isemonger, E.W., Rees, E.R., Dorrington, R.A., Kwan, J.C., 2021. Conserved bacterial genomes from two geographically isolated peritidal stromatolite formations shed light on potential functional guilds. Environ. Microbiol. Rep. 13, 126–137. https://doi.org/10.1111/1758-2229.12916.

Weston, R.A., Perissinotto, R., Rishworth, G.M., Steyn, P.P., 2018. Macroinvertebrate variability between microhabitats of peritidal stromatolites along the South African coast. Mar. Ecol. Prog. Ser. 605, 37–47. https://doi.org/10.3354/meps12741.

Weston, R.A., Perissinotto, R., Rishworth, G.M., Steyn, P., 2019. Benthic microalgal variability associated with peritidal stromatolite microhabitats along the South African coast. Aquat. Microb. Ecol. 82, 253–264. https://doi.org/10.3354/ame01895.

Zhan, A., Hulák, M., Sylvester, F., Huang, X., Adebayo, A.A., Abbott, C.L., Adamowicz, S. J., Heath, D.D., Cristescu, M.E., MacIsaac, H.J., 2013. High sensitivity of 454 pyrosequencing for detection of rare species in aquatic communities. Methods Ecol. Evol. 4, 558–565. https://doi.org/10.1111/2041-210X.12037.