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Unlocking Antarctic molecular time-capsules – Recovering historical environmental DNA from museum-preserved sponges

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Abstract

Marine sponges have recently emerged as efficient natural environmental DNA (eDNA) samplers. The ability of sponges to accumulate eDNA provides an exciting opportunity to reconstruct contemporary communities and ecosystems with high temporal and spatial precision. However, the use of historical eDNA, trapped within the vast number of specimens stored in scientific collections, opens up the opportunity to begin to reconstruct the communities and ecosystems of the past. Here, we define the term 'heDNA' to denote the historical environmental DNA that can be obtained from the recent past with high spatial and temporal accuracy. Using a variety of Antarctic sponge specimens stored in an extensive marine invertebrate collection, we were able to recover information on Antarctic fish biodiversity from specimens up to 20 years old. We successfully recovered 64 fish heDNA signals from 27 sponge specimens. Alpha diversity measures did not differ among preservation methods, but sponges stored frozen had a significantly different fish community composition compared to those stored dry or in ethanol. Our results show that we were consistently and reliably able to extract the heDNA trapped within marine sponge specimens, thereby enabling the reconstruction and investigation of communities and ecosystems of the recent past with a spatial and temporal resolution previously unattainable. Future research into heDNA extraction from other preservation methods, as well as the impact of specimen age and collection method, will strengthen and expand the opportunities for this novel resource to access new knowledge on ecological change during the last century.

KEYWORDS
dried DNA extraction, ethanol DNA extraction, fish diversity, frozen DNA extraction, metabarcoding, Porifera

1 | INTRODUCTION

Environmental DNA (eDNA) surveys have revolutionized how scientists monitor the Earth's marine biome (Takahashi et al., 2023). The capacity to discern biodiversity and ecological processes utilizing genetic material extracted from environmental samples, such as water (Cecchetto et al., 2021), soil (Olmedo-Rojas et al., 2023), sediment (Kuwae et al., 2020), air (Lynggaard et al., 2022), or gut content (Vasiliadis et al., 2024), obviates the necessity for direct species observations, a challenging accomplishment for the inaccessible and vast marine environment (Takahashi et al., 2023). Hence, eDNA metabarcoding surveys detect a significantly larger proportion of the marine biological community compared to traditional approaches, such as diver surveys (Robinson et al., 2023), baited remote underwater video (Stat et al., 2019), and trawling (Llamas et al., 2022). While a partial overlap in species detection is most commonly observed in comparative experiments with traditional monitoring approaches (Robinson et al., 2023), eDNA species detection reliability will further increase when overcoming current limitations, such as enhanced primer design (Wang et al., 2023) and more complete reference databases (Stoeckle et al., 2020). The application of eDNA metabarcoding, therefore, has the potential to increase species detection efficiency while also offering the advantage of non-invasive sampling to reduce potential disturbances to fragile marine ecosystems (Takahashi et al., 2023).

Aquatic eDNA surveys have been observed to achieve high spatial and temporal resolutions (Jensen et al., 2022; Jeunen, Knapp, et al., 2019; Minamoto et al., 2017; O'Donnell et al., 2017), thereby enabling accurate species detection of organisms present near the sampled area. This resolution has been linked to high degradation rates of DNA in the environment and influenced by biotic, e.g., bacterial activity (Tsuji et al., 2017), and abiotic factors including pH and temperature (Strickler et al., 2015; Tsuji et al., 2017). The rapid degradation of eDNA in the open marine environment, however, also limits aquatic eDNA surveys to monitoring contemporary biodiversity patterns (Ramírez-Amaro et al., 2022).

Effective conservation of the marine biome requires current biodiversity trends to be interpreted against accurate historical ecological baselines, allowing an understanding of the magnitude and drivers of past changes (Finnegan et al., 2015; Harnik et al., 2012; Lotze & Worm, 2009). In terrestrial systems, a wealth of historical data has refined our understanding of the changes brought about by direct (Roberts et al., 2017; Wood et al., 2017) and indirect (Parducci et al., 2019; Rick et al., 2013) human pressures. Marine conservation efforts, on the other hand, have only recently begun to use various historical and ancient data sources to determine ecological baselines for the marine environment, such as fossils (Finnegan et al., 2015), midden remains (Seersholm et al., 2018), sediment cores (Finney et al., 2002), and written records (Pauly & Zeller, 2016). Such data sources for the marine biome, however, are extremely scarce (Willis et al., 2007), as well as difficult and expensive to obtain (Kittinger et al., 2015).

Furthermore, information on how marine environments have responded to anthropogenic pressures is mostly incomplete (Hoegh-Guldberg & Bruno, 2010; Kidwell, 2015; Norris et al., 2013). The lack of accurate historical ecological baseline information is particularly pronounced for polar regions, which have suffered profound anthropogenic impacts during the last century through fishing (Pinkerton & Bradford-Grieve, 2014), whaling (Aronson et al., 2011), and climate change (Parkinson, 2019).

Recently, filter-feeding organisms have been investigated as natural eDNA samplers (Junk et al., 2023; Mariani et al., 2019). In particular, marine sponges have been shown to naturally accumulate environmental DNA by continuously filtering large volumes of water to capture particulate matter as a food source (Godefroy et al., 2019). Compared to aquatic eDNA, marine sponges have been observed to hold near-identical vertebrate and eukaryotic diversity patterns within small spatial scales (Jeunen, Cane, et al., 2023; Jeunen, Lamare, et al., 2023), as well as mirroring temporal resolutions in a controlled mesocosm experiment (Cai et al., 2022). Similarly to comparisons between aquatic eDNA and traditional survey approaches, a partial overlap between sponge eDNA and visual surveys has been observed, with sponge eDNA recovering a larger fraction of the fish community in deep-sea and polar regions (Brodnicke et al., 2023; Jeunen et al., 2024). The observed variability in the efficiency of capturing and retaining eDNA signals across species within the phylum Porifera (Brodnicke et al., 2023; Cai et al., 2022) has been linked to microbial activity (Brodnicke et al., 2023). The ability of marine sponges to accumulate eDNA through their filter-feeding strategy enables an exciting opportunity to reconstruct past ecosystems with a previously unattainable temporal and spatial precision by extracting historical eDNA (heDNA) from museum-stored sponge specimens (Neave et al., 2023). We propose the use of the new term "heDNA" to denote the historical environmental DNA that can be obtained from the recent past and enable temporal biodiversity analyses with unprecedented accuracy due to the high temporal and spatial resolution of eDNA in the environment.

While vast numbers of marine sponges have been gathered over centuries for research purposes, various preservation methods have been employed to archive specimens in scientific collections (Ghiglione et al., 2018). For example, within the NIWA Invertebrate Collection (NIC) in New Zealand, marine sponge specimens are most often stored in ethanol, dried, or frozen (Figure 1). A wealth of molecular research aimed at extracting host DNA from museum specimens has revealed preservation techniques to influence DNA degradation rates (Iyavoo et al., 2019; Martínková & Searle, 2006), DNA integrity (Moreau et al., 2013; Zimmermann et al., 2008), and laboratory protocol choice (Hahn et al., 2021; Nagy, 2010; Nishiguchi et al., 2002; Rowe et al., 2011). Hence, to enable heDNA signal comparisons to be made from sponge specimens stored using different preservation techniques, it is essential to understand how preservation method choice impacts and potentially biases heDNA recovery success.

In this study, we determine the feasibility of extracting historical fish eDNA signals from 30 Antarctic sponge specimens stored

FIGURE 1 The number of sponge specimens in the NIWA Invertebrate Collection (NIC) per decade and facetted by preservation method, including dry (yellow), ethanol (blue), frozen (red), formalin (light-grey), isopropanol (grey), and other (dark-grey). Specimens included in 'other' include preservation methods listed as Alcohol Unknown, Ethanol – Previously Unknown, and Slide. Number above bars represent number of specimens. Y-axis reported as square root transformed to increase readability of low-abundant collection numbers. For NIC specimen data, see https://nzobisipt.niwa. co.nz/resource?r=obisspecify.
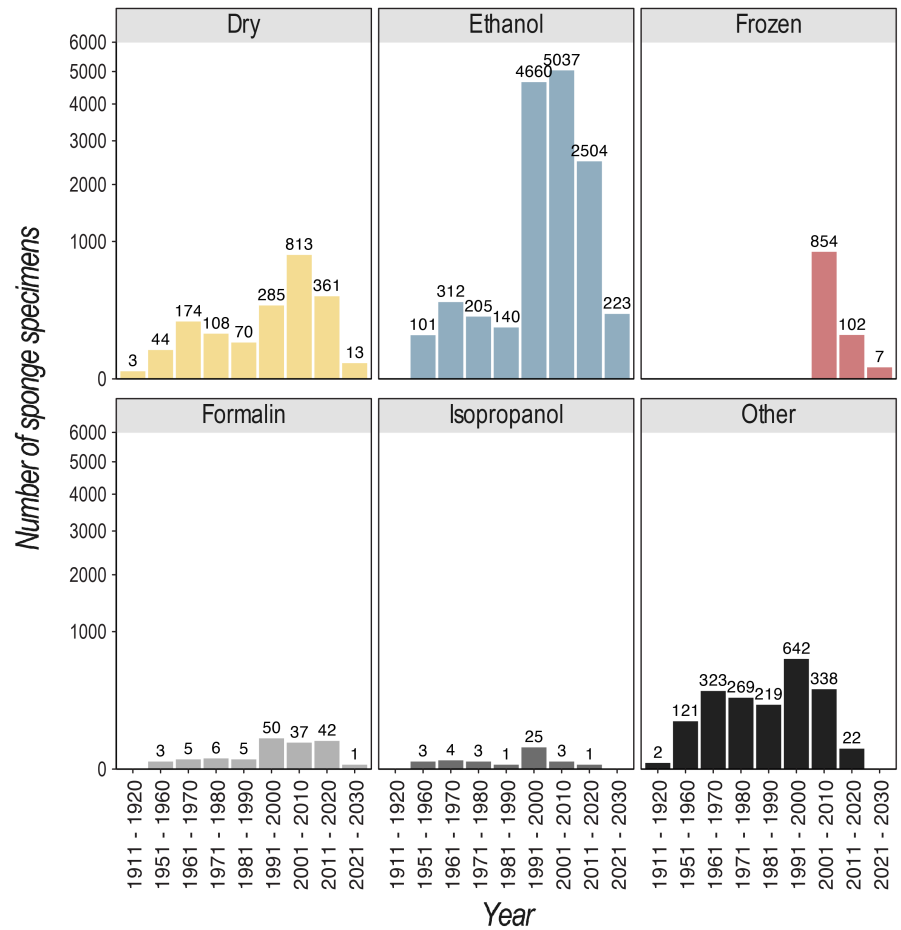
FIGURE 2 Map of the Ross Sea, Antarctica depicting specimen collection locations. Points are coloured by preservation method: Dry (yellow), ethanol (blue), and frozen (red). Point shape is dictated by sponge ID: *Cinachyra* sp. (inverted triangle), *Homaxinella* sp. (circle), *Inflatella belli* (square), *Rossella nuda* (diamond), and *Rossella villosa* (triangle).
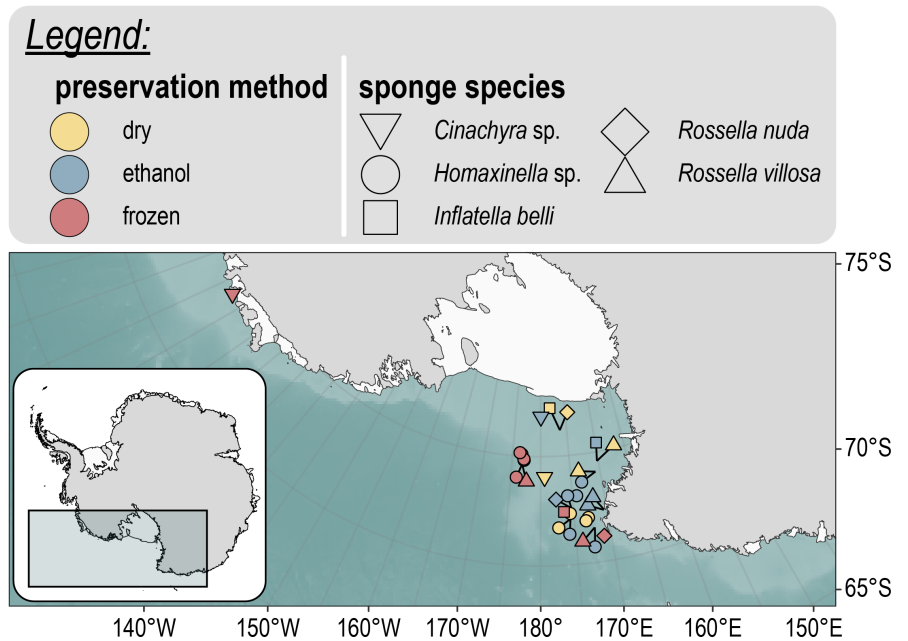
either by ethanol submersion, dried, or frozen (Figure 2). Sponge specimen collection dates ranged from 1960 to 2011. Additionally, we explore the potential bias that preservation methods might introduce to heDNA recovery by comparing alpha and beta diversity metrics from the 30 sponge specimens, while accounting for specimen age and sponge taxonomic ID as potential covariates. Finally, we estimate the replication required to detect 90% of historical fish eDNA signals based on inter- and extrapolation calculations within five tissue biopsy replicates within each sponge specimen.

2 | MATERIALS AND METHODS

2.1 | Museum specimens

We investigated the potential of extracting heDNA from museum-stored sponge specimens preserved using various techniques, including ethanol submersion, dried, and frozen. Within the NIWA Invertebrate Collection (NIC), dried specimens were initially ethanol preserved followed by dry long-term storage, while frozen specimens are a temporary storage solution until long-term specimen preservation in ethanol. Ten specimens from the Ross Sea (Antarctica) were selected for each preservation technique, covering three orders of Demospongiae (Suberitida; Poecilosclerida; Tetractinellida) and the order Lyssacinosida within the class Hexactinellida (Figure 2; Appendix S1). Sponges identified as the same genus, and where possible the same species, were processed for each preservation method to limit the potential effect of eDNA accumulation efficiency differences among sponge species (Brodnicke et al., 2023; Cai et al., 2022). To mitigate the potential variation in successful heDNA recovery due to specimen age, we aimed to process specimens collected around a similar collection date. Hence, all specimens included in this experiment were collected and deposited in the NIC between 2004 and 2010, except for the dried *Cinachyra barbata* Sollas, 1886 specimen from 1960, a species for which no dried specimen from the early 2000s was available.

2.2 | Laboratory processing of sponge specimens

Five tissue biopsies were collected from each specimen at NIC. Biopsies were transported to the University of Otago's PCR-free eDNA facilities at Portobello Marine Laboratory (PML) to minimize contamination risk during sample processing. Bench spaces and equipment were sterilized using a 10-min exposure to 10% bleach dilution (0.5% hypochlorite final concentration) and wiped with ultrapure water (UltraPure™ DNase/RNase-Free Distilled Water, Invitrogen™) before laboratory work (Prince & Andrus, 1992). Additionally, negative controls were processed alongside samples during DNA extraction (50 μL ultrapure water) and added as no template controls during qPCR amplification (2 μL ultrapure water). DNA extractions were performed using the Qiagen DNeasy Blood & Tissue Kit (Cat # 69506; Qiagen GmbH, Germany) following the manufacturer's recommendations, with slight modifications (Appendix S2). DNA extracts were stored at −20°C until further processing.

Input DNA for qPCR amplification was optimized for each sample using a 10-fold dilution series to identify inhibitors and low-template samples prior to library preparation (Murray et al., 2015). Amplification was carried out in 25 μL duplicate reactions. The qPCR mastermix consisted of 1x SensiMIX SYBR Lo-ROX Mix (Cat # QT625-05; Meridian Bioscience, UK), 0.4 μmol/L of the forward [Fish16SF: 5′-GACCCTATGGAGCTTTAGAC-3′ (Berry et al., 2017)]

and reverse [Fish16S2R: 5′-CGCTGTTATCCCTADRGTAACT-3′ (Deagle et al., 2007)] primer (Integrated DNA Technologies, Australia), 2 μL of template DNA, and ultrapure water as required. The thermal profile included an initial denaturation step of 95°C for 10 min; followed by 50 cycles of 30 s at 95°C, 30 s at 54°C, and 45 s at 72°C, and a final melt-curve analysis.

Library preparation followed a one-step amplification protocol using fusion primers (Berry et al., 2017). Fusion primers consisted of an Illumina adapter, a modified Illumina sequencing primer, a 6–8 bp barcode tag, and the template-specific primer (Fish16SF/Fish16S2R) amplifying a ~200 bp fragment of the 16S ribosomal RNA gene region. Each sample was amplified in duplicate and assigned a unique barcode combination, whereby forward and reverse barcodes differed from each other in a single sample. The qPCR conditions followed the protocol as described above. Sample duplicates were pooled to reduce stochastic effects from PCR amplification (Alberdi et al., 2018; Leray & Knowlton, 2015). Samples were pooled into mini-pools based on end-point qPCR fluorescence, C_t-values, and melt-curve analysis. Mini-pools were visualized using gel electrophoresis to confirm the presence of a single band, and the concentration of mini-pools was measured on Qubit (Cat # Q32854; Qubit™ dsDNA HS Assay Kit, ThermoFisher Scientific, US). Equimolar pooling produced a single DNA library. Due to differences in cycle number between samples and negative controls, the latter were spiked into the library to allow for optimal library concentration according to Illumina MiSeq® specifications. Size selection was performed using Pippin Prep (Cat # PIP0001; Sage Science, US). The size-selected library was purified using Qiagen's QIAquick PCR Purification Kit (Cat # 28104; Qiagen GmbH) and quantified using Qubit. Sequencing was performed at the Otago Genomics Facility, University of Otago (New Zealand) on an Illumina MiSeq® instrument using MiSeq reagent kit v2 1x300 bp, with 5%–10% PhiX spiked into the library to minimize issues associated with low-complexity libraries.

2.3 | Bioinformatic analysis and taxonomy assignment

Prior to bioinformatic processing, raw sequencing files were checked for quality using FastQC version 0.11.5 (Andrews, 2010). Reverse Illumina adapter sequences, present due to the amplicon size being smaller than the sequencing kit cycle number, were removed from reads using cutadapt version 4.1 (Martin, 2011) without allowing indels. Reads were demultiplexed and assigned to samples using cutadapt, allowing for two mismatches in the barcode and primer region. The assigned amplicons were filtered using the '--*fastq_filter*' function in VSEARCH version 2.13.3 (Rognes et al., 2016) based on a maximum expected error of 1.0, a minimum length of 190 bp, a maximum length of 220 bp, and without allowing the occurrence of ambiguous base calls. The remaining reads were checked for successful quality filtering using FastQC before dereplication (function: '*vsearch --derep_fulllength*'). Chimeric sequences were removed and Zero-radius Operational Taxonomic Units (ZOTUs) were generated

using the '-unoise3' function (Edgar, 2016) in USEARCH version 11.0.667 (Edgar, 2016). Finally, a frequency table was generated using the '-otutab' function in USEARCH.

A custom-curated reference database was generated using CRABS version 0.1.5 (Jeunen et al., 2022). The custom-curated reference database consisted of sequences downloaded from multiple online repositories using the '*db_download*' function and *in-house* generated barcodes of Southern Ocean fish species (Jeunen et al., 2024) using the '*db_import*' function. Amplicon regions were extracted from sequences through in silico PCR analysis ('*insilico_pcr*' function) and pairwise global alignments ('*pga*' function). Finally, the curated reference database was filtered (function: '*seq_cleanup*') and dereplicated (function: '*dereplicate*'). The final reference database was formatted according to IDTAXA specifications (Murali et al., 2018) and used as the reference database (Appendix S3) to train the IDTAXA classifier through five iterations using the '*LearnTaxa*' function in the DECIPHER R package (Wright, 2016). Finally, all ZOTU sequences were classified using the '*IdTaxa*' function in DECIPHER, with the recommended default confidence threshold of 60% as the cut off value to determine the taxonomic ID level. Sequences for which no taxonomic ID could be achieved at the order level with the 60% cut off threshold were BLASTed against the full NCBI database.

After taxonomy assignment, the frequency table underwent final processing before statistical analysis, whereby (i) detections were only kept when reaching a read count higher than the most abundant detection in the summed negative controls, (ii) sequences with a positive detection in the negative controls were deemed true detections in samples when achieving a 10x read count compared to the negative controls, (iii) sequences were removed from the final data set if no taxonomic ID could be obtained for at least the order level, (iv) non-Antarctic taxonomic IDs were removed from the frequency table, (v) artefact sequences were merged with their parent based on taxon-dependent co-occurrence patterns of similar sequences, and (vi) samples not reaching a total abundance of 10,000 reads were removed from the analysis.

2.4 | Statistical analysis and visualization

Statistical analyses and visualizations were conducted in R version 4.0.5 (R; http://www.R-project.org) unless specified otherwise. Rarefaction curves were generated from the unfiltered frequency table to assess sequencing coverage using the *vegan* version 2.5-7 package (Dixon, 2003). Species accumulation curves were drawn for Hill numbers of order q: species richness ($q=0$), the exponential of Shannon entropy ($q=1$), and the inverse of Simpson concentration ($q=2$) to assess replication coverage per specimen using the iNEXT.3D version 1.0.1 R package (Chao et al., 2021). Summary statistics on the read count and most abundant taxa were obtained through the phyloseq version 1.44.0 (McMurdie & Holmes, 2013) and microbiome version 1.23.1 R packages. To assess alpha diversity differences among preservation methods, the frequency table was transformed to an incidence-frequency data set. Hill numbers

of orders $q=0$, 1, and 2 were compared among preservation methods through a one-way ANOVA. Taxonomic diversity estimates for Hill order $q=0$ were calculated through inter- and extrapolation in iNEXT.3D (function: '*estimate3D*') to assess the required replication at 90% coverage for each specimen. Significant differences among preservation methods for the required replication were tested through a one-way ANOVA, followed by post hoc Fisher's LSD (Least Significant Difference). Non-metric MultiDimensional Scaling (NMDS) ordination plots were drawn using the phyloseq function '*ordinate*' to examine beta diversity patterns. Statistical significant differences in beta diversity among preservation methods, sampling methods, sponge IDs, depth, latitude, and longitude were tested through PERMANOVA (function '*adonis2*') and PERMDISP analyses ('*betadisper*'). Bioinformatic and R scripts and metadata files can be found on the GitHub repository https://github.com/gjeunen/marsden_obj1_preservationMethod. The raw sequence data are deposited onto the NCBI short read archive (SRA) under project number PRJNA1019816.

3 | RESULTS

3.1 | High-throughput sequencing results

Demultiplexing of raw sequencing data assigned 10,989,938 sequences to heDNA extracts. Quality filtering and denoising returned a total of 10,680,592 (97.19%) sequences assigned to 153 ZOTUs. Post-processing identified four reads in negative controls, including three reads assigned to ZOTU 2 (*Macrourus* sp.) and one read assigned to ZOTU 8 (*Pleuragramma antarcticum* Boulenger, 1902). Hence, all detections with three reads or lower were discarded from the frequency table, as well as detections with 30 reads or lower and 10 reads or lower for ZOTU 2 and ZOTU 8, respectively. IDTAXA failed to provide a taxonomic ID at the order level for 38 ZOTUs (10,244 reads). As no high-quality BLAST-hits were achieved for these 38 ZOTUS, all 38 ZOTUs were removed from the analysis. Additionally, three ZOTUs were assigned to temperate taxa and removed from the analysis, including ZOTU 56 (taxonomic ID: Cheilodactylidae; read abundance: 2203; detections: PMD7b), ZOTU 71 (taxonomic ID: *Helicolenus* sp.; read abundance: 582; detections: PMD7e), and ZOTU 88 (taxonomic ID: *Thyrsites atun* (Euphrasen, 1791); read abundance: 131; detections: PMF3e, PMF9a). After merging artefact sequences, 64 ZOTUs were retained for the final analysis. Nine samples did not obtain a read count of 10,000 sequences, including PMD1d and multiple samples belonging to the sponge genus *Cinachyra* spp. irrespective of the preservation method used (PMD4; PME3; PMF5). Therefore, all samples belonging to genus *Cinachyra* were removed from the analysis. Post-processing of the frequency table retained a total of 9,829,826 (92.03%) reads for statistical analysis (Appendix S4). Overall, samples achieved sufficient sequencing coverage based on the plateauing of rarefaction curves (Appendix S5) and mean number of reads per sample $\pm$ SD: 73,357 $\pm$ 25,183.

3.2 | Alpha diversity measurements

Post-processing returned 64 ZOTUs for which a taxonomic ID could be achieved, covering 25 families, 15 orders, and 2 classes (Figure 3; Appendix S4). The Antarctic toothfish (*Dissostichus mawsoni*, Norman, 1937) was the most abundant signal across all samples (sequence ID: ZOTU 1; read count: 2,318,043; proportional

abundance: 23.58%), followed by the genus *Macrourus* (sequence ID: ZOTU 2; read count: 1,877,328; proportional abundance: 19.10%) and the Antarctic silverfish (*Pleuragramma antarcticum*; sequence ID: ZOTU 3; read count: 1,856,736; proportional abundance: 18.89%). The Antarctic toothfish was also the most frequently detected species across all samples (detections: 128/134), followed by the Antarctic silverfish (detections: 122/134) and cod
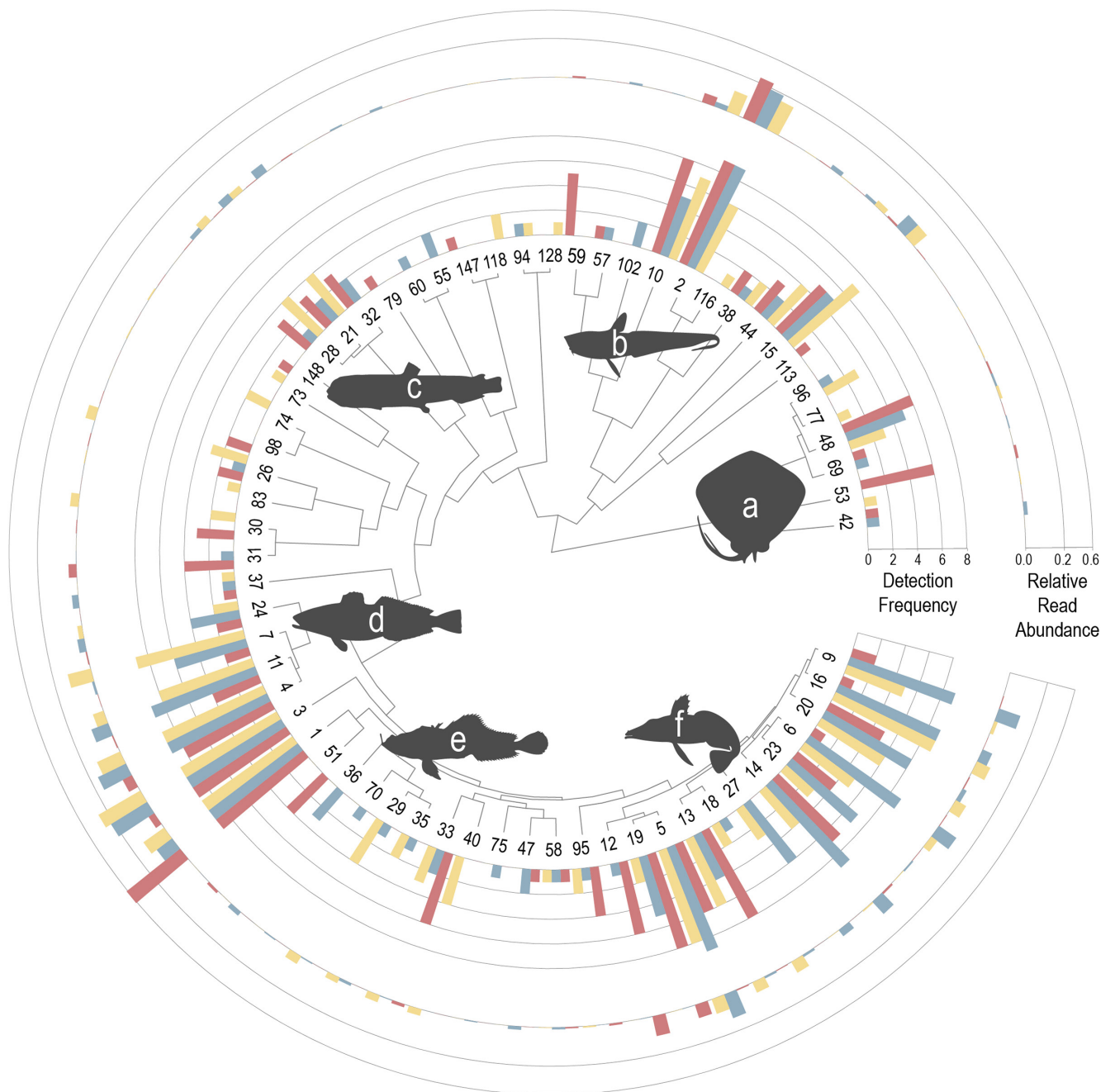
FIGURE 3 Bayesian phylogenetic tree generated for all 64 ZOTU sequences. Tip labels represent ZOTU number. Taxonomic ID for each ZOTU can be retrieved from Supplement 4. Inner bar graph showing the number of detections of each ZOTU sequence within the nine specimens stored dry (yellow), in ethanol (blue), and frozen (red). Outer bar graph showing the relative read abundance of each ZOTU sequence within the nine specimens stored dry (yellow), in ethanol (blue), and frozen (red). Axis for relative read abundance bar graph is reported as square root transformed to increase readability of low-abundant signals. Most frequent and abundant taxonomic groups are represented by silhouettes, including (a) Chondrichthyes, (b) Gadiformes, (c) Bathylagidae, (d) Nototheniidae, (e) Bathydraconidae, and (f) Channichthyidae.

icefish in the genus *Trematomus* (sequence ID: ZOTU 4; detections: 106/134).

Five DNA extracts per sponge specimen were deemed sufficient to recover most of the fish diversity held within the sponge according to the plateauing of species accumulation curves (Appendix S6). The estimated replication needed to recover 90% of the fish diversity, based on inter- and extrapolation calculations, differed significantly among preservation methods according to a one-way ANOVA ($F_{2,23} = 3.463$, $p = .048^*$) when removing the outlier sample PMD1 (data from only 4/5 replicates, with 1/5 replicates dropped out). Fisher's LSD identified frozen specimens (8.840 ± 4.102) to be significantly different from ethanol-stored (5.116 ± 3.224) and dried (5.215 ± 2.579) specimens (Figure 4a). Without removing the outlier sample, no significant differences among preservation methods were observed according to a one-way ANOVA ($F_{2,24} = 1.893$, $p = .173$; Appendix S7). Alpha diversity investigations among preservation methods yielded no significant differences across three orders of Hill numbers according to one-way ANOVA ($q = 0$: $F_{2,24} = 0.146$, $p = .865$; $q = 1$: $F_{2,24} = 0.237$, $p = .791$; $q = 2$: $F_{2,24} = 0.444$, $p = .647$; Figure 4b).

3.3 | Community composition analyses

Significant differences were observed in community composition among preservation methods according to PERMANOVA ($F_{2,26} = 2.294$; $R^2 = .129$; $p < .005^*$), while sampling method ($F_{2,26} = 1.335$; $R^2 = .075$; $p > .1$) and sponge ID ($F_{3,26} = 1.165$; $R^2 = .098$; $p > .1$) were found non-significant explanatory variables. However, the largest fraction of the variability in the data set was

left unexplained (Residual $R^2 = .533$). No significant differences in dispersion were detected among preservation methods according to PERMDISP ($F_{2,26} = 1.936$; $p > .1$), indicating PERMANOVA significance resulted from different centroid position in ordination space. Historical fish eDNA signal differences were confirmed by ordination analysis (NMDS; Bray-Curtis index; frequency-occurrence transformation; stress = 0.171; Figure 5), whereby the differently coloured preservation methods and filled versus outline for sampling methods highlight the confounding factors of frozen specimens collected through commercial longlining and dried and ethanol-stored specimens collected by scientific trawling.

4 | DISCUSSION

Environmental DNA biomonitoring has helped increase our understanding of biodiversity and, ultimately, ecosystem functioning (Aglieri et al., 2021; Seymour et al., 2021). Thus far, eDNA has been applied to a range of habitats and locations (Ruppert et al., 2019). Furthermore, the ease of sample collection to monitor biodiversity across the tree of life has made eDNA especially beneficial for remote and logistically demanding environments that are spatially and temporally under-sampled, such as the Antarctic (Clarke et al., 2023; Howell et al., 2021). Obtaining quantitative spatial and temporal information on Antarctic species is more than ever critical, with the region forecast to see major physical and biological changes in response to climate change and anthropogenic pressures (Chown & Brooks, 2019; Convey & Peck, 2019). While eDNA has been successfully implemented to monitor contemporary biodiversity patterns of
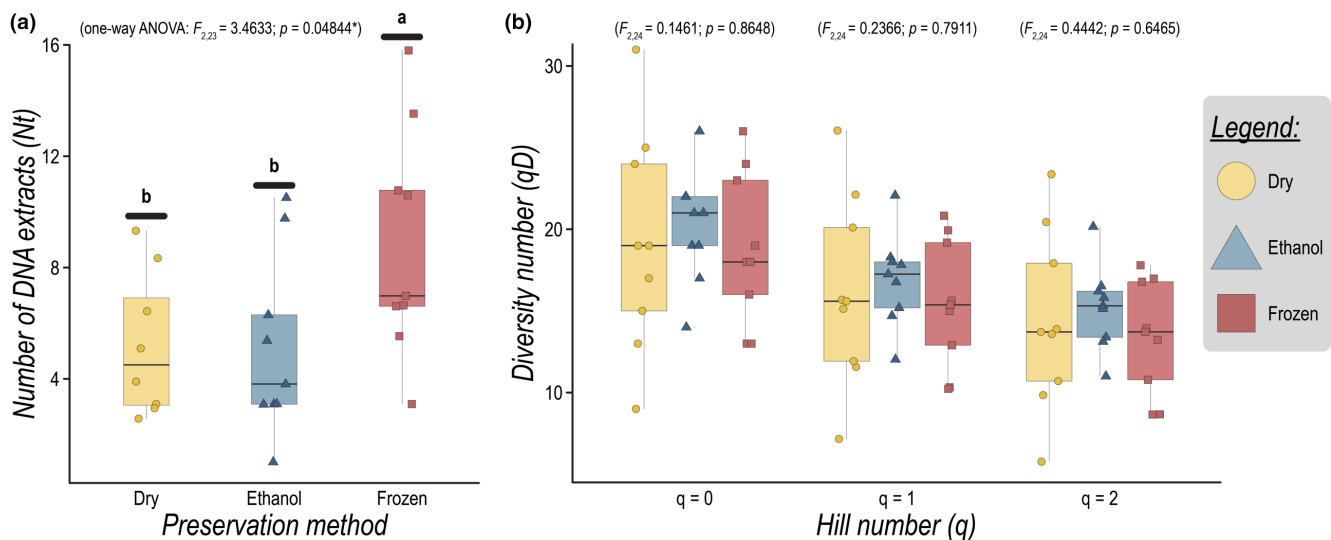

FIGURE 4 (a) Boxplots depicting the estimated tissue biopsies needed to recover 90% of the fish diversity among the three preservation methods, including dry (yellow), ethanol (blue), and frozen (red). The median is indicated by a black line within each boxplot. Samples are indicated by coloured dots, including circle (dry), triangle (ethanol), and square (frozen). The outlier specimen PMD1 (four out of DNA extracts yielded fish eDNA signals) was removed from the analysis. One-way ANOVA results are presented above the figure. Significant differences among preservation methods, as reported by Fisher's LSD, are indicated by lower-case letters. (b) Boxplots depicting alpha diversity measurements among the three preservation methods for three orders of Hill numbers, including $q = 0$ (species richness), $q = 1$ (exponential of Shannon entropy), and $q = 2$ (inverse of Simpson concentration). Non-significant one-way ANOVA results are presented above the figure. Colour and shape follow Figure 4a.
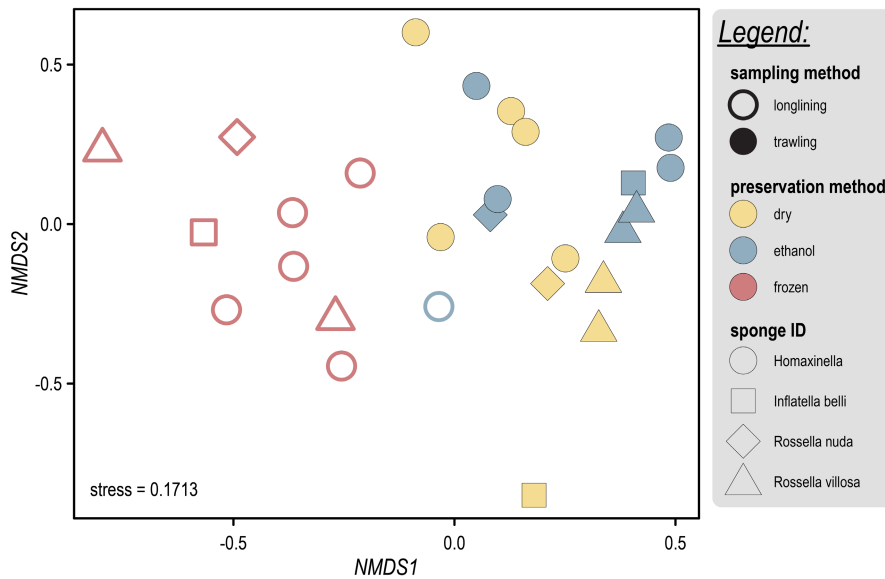
FIGURE 5 Non-metric multi-dimensional scaling (NMDS) plot depicting similarity in fish community composition based on occurrence frequency (Bray-Curtis index; frequency count). The stress value is reported in the lower left-hand corner. Points are coloured according to preservation method: Dry (yellow), ethanol (blue), and frozen (red). Shape is dictated by sponge ID, with *Homaxinella* sp. represented as circles, *Inflatella belli* as squares, *Rossella nuda* as diamonds, and *Rossella villosa* as triangles. Filled shapes indicate sponge specimens collected through trawling. Outlined shapes indicate sponge specimens collected through longlining.

the Antarctic marine biome (Clarke et al., 2021; Cowart et al., 2018; Jeunen, Lamare, et al., 2023; Liao et al., 2023; Suter et al., 2023), a lack of long-term, quantitative observations limits our understanding of the natural variability in Antarctic ecosystems and complicates future policymaking (Howell et al., 2021; Suter et al., 2023). Hence, investigating historical and ancient DNA has the potential to provide the missing information for successful conservation efforts in Antarctica.

In this study, we provide evidence for a widely available but previously untapped resource of historical ecological data that takes advantage of the natural accumulation of eDNA in filter-feeding tissue matrices (Mariani et al., 2019; Neave et al., 2023). Using a targeted metabarcoding approach, we successfully recovered the historical fish eDNA accumulated within Antarctic sponge specimens. Successful DNA extraction from specimens stored using three common preservation techniques, i.e., ethanol submersion, drying, and freezing, increases the number of specimens available for analysis. With vast numbers of marine sponges having been gathered globally since the earliest scientific voyages (Wulff, 2016), these archived specimens provide unique ecosystem time capsules through which we can reconstruct historical biodiversity patterns and provide essential knowledge for current conservation efforts (Revéret et al., 2023).

We were able to identify a diverse profile of Actinopterygii and Chondrichthyes from Antarctic sponge specimens, irrespective of the preservation method used. The Antarctic fish community constituted 64 taxa ranging from Nototheniidae (cod icefishes) and Channichthyidae (icefishes), to Bathydraconidae (Antarctic dragonfishes), all of which are known to occur in the Ross Sea according to Antarctic toothfish bycatch records (Jeunen et al., 2024; Pinkerton & Bradford-Grieve, 2014). Interestingly, one notably absent taxonomic group, besides two signals of *Gymnoscopelus* sp., from sponge specimens collected in deeper waters are the myctophids, the most diverse and abundant group of mesopelagic fishes globally, including in the Southern Ocean (Duhamel et al., 2014; Vasiliadis et al., 2024; Woods et al., 2023). The lack of myctophid

detection could potentially have stemmed from their occupancy of the mesopelagic zone (Catul et al., 2011; Christiansen et al., 2018). The vertical distance between myctophids and benthic sponges is known to influence eDNA metabarcoding detection results (Jeunen, Lamare, et al., 2019). Additionally, multiple mismatches at the 3′ end of the forward PCR primer-binding region (Appendix S8) could have significantly reduced the amplification efficiency for this taxonomic group, resulting in false-negative detections (Stadhouders et al., 2010). While universal metabarcoding approaches have been reported to be an inefficient solution due to the co-amplification of sponge host DNA (Jeunen, Lamare, et al., 2023), a multi-marker targeted metabarcoding approach has previously been proposed for aquatic eDNA research to increase species detection accuracy and reduce the impact of amplification bias (McElroy et al., 2020).

Our results provide evidence for the importance of accurate metadata to interpret observed biodiversity patterns and gauge the potential impact of biases in species detection from eDNA metabarcoding. For example, the taxonomic group to which a sponge belongs has been identified in previous research (Brodnicke et al., 2023; Cai et al., 2022), as well as here, to impact eDNA detection success. In our study, specimens from the genus *Cinachyra* failed to reliably amplify fish eDNA signals, irrespective of the preservation technique used to store the specimens. Additionally, a significant difference in the reported fish community was observed among preservation methods. This difference, however, could have originated from the confounding factors of collection location and method. Namely, frozen specimens were collected by commercial Antarctic toothfish longlining vessels located further offshore compared to dried and ethanol-stored specimens collected by scientific trawling along the Ross Sea coastline.

Thus far, contemporary sponge eDNA research has focused on single-tissue biopsies for eDNA signal detection (Brodnicke et al., 2023; Cai et al., 2022; Mariani et al., 2019; Neave et al., 2023). However, replicate biopsies collected from a single sponge specimen combined with rarefaction and extrapolation of species diversity identified the need to collect between five (dried and

ethanol-stored) and nine (frozen) biopsies per specimen to confidently detect 90% of the fish diversity held within marine sponges. While multiple tissue biopsies from each sponge increase the overall cost of the project and may not be possible for small and/or rare specimens in collections, replication enables data transformation to frequency-occurrence (Chao et al., 2021), thereby providing semi-quantitative, i.e., incidence-based, data and expanding upon the statistical analyses able to be conducted (Alberdi & Gilbert, 2019). The need for increased replication likely stems from a lack of understanding about the process of eDNA accumulation in the sponge tissue matrix. Further research into laboratory protocol development to efficiently extract eDNA from sponge tissues (Harper et al., 2023), as well as gaining a better understanding of eDNA accumulation by sponges (Cai et al., 2022), are essential to progress the applicability of sponges as natural eDNA samplers. Our results show significant differences in estimated replication between treatments, with frozen samples requiring increased tissue biopsies to reliably detect 90% of the fish diversity within a specimen compared to dried and ethanol-submerged specimens. The significant difference in the required replication could have been induced by the highly dominant signal of *D. mawsoni*, the target fish of the longlining fishing vessels from which frozen specimens were collected, thereby reducing the detection probability of the remaining low-abundant fish eDNA signals (Bylemans et al., 2019; Ficetola et al., 2015; Rojahn et al., 2021).

The challenge in verifying species detection became evident from the presence of temperate fish species in our dataset. All temperate fish species were conspicuously absent in the negative control samples, thus unlikely to be a result from internal lab contamination. The power and sensitivity of present-day molecular approaches require high standards to minimize the risk of DNA contamination in the field and throughout curation and laboratory handling (Goldberg et al., 2016; Llamas et al., 2017). Processing ancient and historical specimens, most of which were not collected nor handled for molecular analysis purposes throughout the time stored in scientific collections, increases the risk of DNA contaminants being incorporated into the specimens through, for example, (i) cross-contamination from handling multiple specimens without bench-space and equipment sterilization, or (ii) transferring specimens and fixatives between collection lots (Cowart et al., 2022; Knapp et al., 2012). For ancient DNA shotgun sequencing approaches, DNA damage profiles can be assessed to identify modern DNA contaminants (Seersholm et al., 2016). However, when utilizing historical metabarcoding techniques, DNA damage profiles cannot be successfully implemented for contaminant identification (Piper et al., 2019). Within eDNA metabarcoding and microbiome research, removal of contaminants has been largely based on abundance filtering (Li et al., 2018), detection frequency filtering (Evans et al., 2017), and removal of non-target species (Alberdi et al., 2018), as employed in this study.

The selection of preservation methods included in this study was determined by identifying the techniques with the highest number of sponge specimens within NIC. Exploring additional common curation methods, such as formalin fixation (Hykin et al., 2015; Srinivasan et al., 2002), will further increase the pool of available specimens for

historical eDNA research. Genetic and genomic investigations utilizing formalin-fixed museum specimens have been challenging in the past, since formaldehyde reduces DNA integrity and produces sequence artefacts by inducing numerous molecular lesions, such as strand breaks, base misincorporation, and intra- and intermolecular cross-linking (Do & Dobrovic, 2015; Srinivasan et al., 2002; Williams et al., 1999). However, recent advances in whole-genome sequencing of formalin-fixed paraffin-embedded (FFPE) archival tissues (Robbe et al., 2018; Stiller et al., 2016) and formalin-fixed museum specimens (Hahn et al., 2021) provide a tantalizing prospect to explore formalin-fixed sponge specimens for historical eDNA research, which we will seek to undertake in future studies.

To fully utilize the power of this novel historical resource, we propose three future research avenues. First, while our results provide evidence for successful heDNA extraction following multiple preservation techniques, investigations into optimal storage methods and associated biases require specimens to be divided and preserved in various ways (Spens et al., 2016). Such information will guide scientists in choosing optimal specimens for heDNA research (Hahn et al., 2021) and set storage standards for building future resources. Second, to minimize the number of covariates in this study, we aimed to incorporate specimens from a similar collection date range in the experiment. Further investigations into the effect of specimen age for each preservation technique would provide useful information on the utility of older specimens. While the dried *C. barbata* specimen from 1960 failed to amplify historical fish eDNA signals, the result was most likely influenced by sponge taxonomy rather than age, as we successfully amplified and analysed the oldest Antarctic sponge specimen stored in ethanol (collection date: 1958) at the NIC for fish eDNA signals (GJ. Jeunen, personal communication). Third, museum specimens are precious but finite resources for scientific research (Hahn et al., 2020). Therefore, minimizing the destruction of valuable voucher specimens is essential and will require the use of optimized wet lab protocols, as well as investigations into non-destructive DNA extraction approaches, such as direct heDNA extraction from preservative medium rather than tissue biopsies (Rohland et al., 2004; Shokralla et al., 2010).

5 | CONCLUSION

Marine environments and species have been exploited throughout human history, leading to entire ecosystem modification, habitat degradation, and multiple species extinctions. Therefore, mitigation and restoration of degraded marine systems is of top global economic, ecological, and cultural importance. However, successful remediation requires detailed knowledge of how these ecosystems have altered over time. Currently, the extent and speed of ecological change in the marine domain have rarely been quantified because long-term ecological records are scarce and accurate historical data are difficult and expensive to obtain. In this experiment, we provide evidence for using the historical eDNA trapped within taxonomic collection sponge specimens as a novel ecological record source to investigate historical

biodiversity patterns at a previously unattainable temporal and spatial scale. The successful recovery of historical eDNA from sponge specimens stored using various preservation techniques significantly broadens the pool of specimens to be included in this type of research. Future investigations into the impact of additional preservation techniques such as formalin-fixation, as well as specimen age, and collection method are essential to fully utilize this novel methodology.

AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Bioinformatic and R scripts, as well as metadata files, can be found on the GitHub repository https://github.com/gjeunen/marsden_obj1_preservationMethod and available as a Zenodo release (DOI: 10.5281/zenodo.12654989). The raw sequence data are deposited onto the NCBI short read archive (SRA) under project number PRJNA1019816.

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SUPPORTING INFORMATION

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