Ancient DNA and microfossils reveal dynamics of three harmful dinoflagellate species off Eastern Tasmania, Australia, over the last 9,000 years

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Highlights

- Dinocyst and *sed*aDNA analyses were applied to marine sediments off Tasmania
- *Alexandrium catenella* has been endemic to Australia for at least ~9,000 years
- Recent *A. catenella* blooms are likely induced by climate and oceanographic change
- *Gymnodinium catenatum* cysts in recent (~30y) sediments confirm a 1970s introduction
- *Noctiluca scintillans sed*aDNAin recent (~30y) sediments matches a 1994 introduction

Abstract

Harmful algal blooms (HABs) have significantly impacted the seafood industry along the Tasmanian east coast over the past three decades, and are expected to change in frequency and magnitude due to climate change induced changing oceanographic conditions. To investigate the long-term history of regional HABs, a combination of palynological and sedimentary ancient DNA (*sed*aDNA) analyses was applied to marine sediment cores from inshore (up to 145 years old) and offshore (up to ~9,000 years) sites at Maria Island, southeast Tasmania. Analyses focused Paralytic Shellfish Toxin (PST) producing dinoflagellates *Alexandrium catenella* and *Gymnodinium catenatum,* and the red-tide

dinoflagellate *Noctiluca scintillans*, which were specifically targeted using a hybridization capture *sed*aDNA technique. Identification of primulin-stained *A. catenella* cysts throughout the inshore sediment core, together with *sed*aDNA evidence of a bloom-phase of *Alexandrium* ~15 years ago, indicates recent stimulation of a cryptic endemic population. Morphologically similar but unstained *Alexandrium* cysts were observed throughout the offshore core, with *sed*aDNA confirming the presence of *A. catenella* from ~8,300 years ago to present. *Gymnodinium catenatum* cysts were detected only in inshore surface sediments from 30 years ago to present, supporting previous evidence of a 1970s introduction via shipping ballast water. *sed*aDNA confirmed the presence of *G. catenatum*-related sequences in the inshore and offshore cores, however, unambiguous species identification could not be achieved due to limited reference sequence coverage of *Gymnodinium*. Our hybridization capture *sed*aDNA data also confirmed the historically recent dispersal of the non-fossilizing dinoflagellate *Noctiluca scintillans*, detected inshore from ~30 years ago, matching first observations of this species in Tasmanian waters in 1994. At the offshore site, *N. scintillans sed*aDNA was detected only in surface sediments, confirming a recent climate-driven range expansion this species. This study provides new insights into the distribution and abundance of three HAB species in the Tasmanian region, including clues to past bloom phases. Further research into paleo-environmental conditions and paleo-community structure are required to identify the factors driving bloom phases through time and predict plankton community responses under different future climate scenarios.

Key words: dinoflagellate; biotoxin; ballast water; ancient DNA; seafloor; Australia

Abbreviations: harmful algal bloom, HAB; heuristic operations for pathogen screening, HOPS, hydrofluoric acid, HF; internally transcribed spacer, ITS; sedimentary ancient DNA, *sed*aDNA; small subunit ribosomal rRNA, SSU; large subunit ribosomal rRNA (LSU)

1 Introduction

The issue of whether harmful algal blooms (HABs) are increasing in extent and frequency due to climate change and the potential role of eutrophication and anthropogenic transport, remain hotly debated topics (Hallegraeff et al., 2021). When novel bloom phenomena impact on tourism, aquaculture, fisheries and human health, questions arise as to whether these blooms are the result of a recent species introduction into the area or of a cryptic endemic species' massive growth that is newly stimulated by changing environmental conditions. Much research has been undertaken deciphering the association of new algal bloom phenomena with human-assisted ballast water introductions (McMinn et al., 1998) or extreme climate events (Trainer et al., 2019). In contrast, very few studies have attempted to prove the 'always-been-there, but previously-overlooked' scenario. To date, only a few studies have analysed long-term, *i.e.*, thousands of years, dynamics of harmful algal bloom species or the timing and mode of introduction to a region (*e.g.*, Thorsen et al., 1995, for *Gymnodinium*

nolleri (as *G. catenatum*) in Scandinavia; Klouch et al., 2016, for *Alexandrium minutum* in the Bay of Brest).

The ocean environment off eastern Tasmania is well documented as a climate change hotspot, characterized by a strengthening East Australian Current and rapidly increasing ocean temperatures (2.3 ℃ increase since the 1940s, Ridgway and Hill, 2009). The consequences of this rapid change are now being detected in coastal marine communities, including changes in plankton and HAB species composition (Thompson et al., 2009, Condie et al., 2019). A planktonic HAB example is the dinoflagellate *Gymnodinium catenatum*, a Paralytic Shellfish Toxin (PST) producer thought to be introduced to Tasmania in the 1970s by shipping ballast water. This species appeared noticeably in the mid-1980s, causing PST contamination up to 250x acceptable limits and extensive shellfish farm closures in the Derwent-Huon estuaries from 1986 to 1993. After most shellfish farming in the area went bankrupt, the local government declared the area unsuitable for bivalve aquaculture. Supporting evidence for its introduction includes cysts detected in ships ballast tanks (Hallegraeff and Bolch, 1992), cysts evidence from marine sediment core analysis (McMinn et al., 1998), reproductive compatibility studies (Blackburn et al., 2001) and molecular genetic evidence (Bolch and de Salas, 2007).

The regional appearance and increase of other HAB phenomena have in contrast been attributed to climate-driven range expansion; exemplified by the dinoflagellate *Noctiluca scintillans*. Highly visible red tides and bioluminescent spectacles caused by *Noctiluca* were first documented in Australia from Sydney Harbour in 1860 (Bennett, 1860), have been more frequently reported since the 1990s, and even caused temporary closure of popular Sydney tourist beaches (Hallegraeff et al., 2020). Amongst the causes of *N. scintillans* blooms are eutrophication, upwelling and ocean circulation (Dela Cruz et al., 2002, 2003). *Noctiluca* was first observed in Tasmania in 1994, carried south by the East Australian Current, and representing a new threat to the expanding salmonid fish farm industry in 2002 (Hallegraeff et al., 2019). Finally, in 2010 the organism appeared to have moved into the Southern Ocean for the first time (240 km south of Tasmania), raising concerns about grazing impacts on iconic krill-based food webs (McLeod et al., 2012).

More seriously, starting in 2012, the Tasmanian seafood industry experienced industry closures affecting bivalves, abalone, rock lobster, and public health warnings associated with Paralytic Shellfish Poisoning (up to 150 mg STX eq./kg) during winter-spring blooms of the cold-water dinoflagellate *Alexandrium catenella* (Condie et al., 2019). The morphologically identical but genetically distinct species A. *australiense* and *A. pacificum* (Bolch and DeSalas, 2007) were known from Tasmanian waters, but the presence of *A. catenella* (=*Alexandrium tamarense* Group 1; John et al., 2014), had never been previously detected. Tasmanian cultured populations are known to possess a unique microsatellite DNA signature different from other global populations, arguing that *A. catenella* has been present prior to European settlement in 1788 - 1803 and thus represents an endemic cryptic population stimulated by changing environmental conditions, such as increasing winter water column stratification (Trainer et al., 2019). At least five cases of non-fatal human paralytic shellfish

poisonings (one from *G. catenatum* and four from *A. catenella*) have been formally reported from Tasmania in the past 10 - 20 years, but it is unclear whether seafood poisonings may have occurred throughout longer-term (thousands of years) Tasmanian history (Jones, 1978).

To gain insights into long-term dynamics of harmful dinoflagellate species, their introduction into, and disappearance and reappearance in a region, marine sediment archives are a significant resource. After phytoplankton (including dinoflagellates) die or form resting stages/cysts following unfavourable conditions, they sink to the seafloor where they accumulate in layers, so that over time their fossil assemblages form a geological record of past presence and abundance. However, such microfossil assemblages can only provide part of the picture, as only the most robust species make it to the seafloor, and soft-bodied species are missing entirely from the sediment record. For example, While *Gymnodinium catenatum* produces cysts that fossilize (Anderson et al., 1988), the cysts of *Alexandrium catenella* have only limited durability in the sediment record (Head et al., 2006), and *Noctiluca* does not produce a cyst at all. Additionally, individual species of varying toxicity within the *A. tamarense* species complex cannot be distinguished based on either planktonic cell or cyst morphology.

Recently, sedimentary ancient DNA (*sed*aDNA) analyses have been increasingly used as a tool to characterise past marine ecosystems from seafloor sediments over geological timescales. These novel *sed*aDNA analyses offer great potential to overcome the hurdle of accessing information on less-well preserved species, and gain insights into paleo-communities across all domains of life, including, phyto- and zooplankton (Armbrecht, 2020). Indeed, preliminary DNA analyses on seafloor surface sediments collected near Maria Island (Spring Bay), Tasmania, first pointed to the possibility of retrieving ancient DNA preserved from all three HAB species in this region (Shaw et al., 2019). Since then, *sed*aDNA extraction techniques and bioinformatic analyses have been refined and optimized to detect, isolate and analyse the minuscule amounts of eukaryote *sed*aDNA preserved in marine sediments (Armbrecht et al., 2020, 2021). Particularly promising is the application of hybridization capture techniques to *sed*aDNA, where short RNA probes ("baits") capture complementary DNA fragments in a DNA extract (Horn et al., 2012), an approach enabling the study of specific genes and/or organisms of interest. This 'bait' approach, recently applied to capture marine eukaryote *sed*aDNA (Armbrecht et al., 2021), seems highly suitable to the detailed study of past dinoflagellate dynamics and blooms of both cyst forming and non-cyst forming species.

We here combined palynological and *sed*aDNA techniques (including hybridization capture) to resolve the dynamics of the three HAB species *Alexandrium catenella, Gymnodinium catenatum* and *Noctiluca scintillans* over the past ~9,000 years. Our aim was to address the following questions: (1) Is there evidence from the sediment record that these three HAB species are endemic to eastern Tasmania? (2) Has their abundance changed significantly over that period? (3) What are the characteristics (*e.g.*, DNA damage and fragment size) of *Alexandrium, Gymnodinium* and *Noctiluca sed*aDNA preserved in coastal marine sediments off Eastern Tasmania, and how do these characteristics influence the predicted maximum

timescales of harmful dinoflagellate *sed*aDNA preservation and detection at this coring location?

2 Materials and Methods

2.1 Sediment Core Collection and Preparation

An approximately 3 m long marine sediment core (gravity core, designated 'GC2S1') was collected in May 2018 during *RV Investigator* voyage IN2018_T02 in 104 m water depth close to the continental shelf edge, east of Maria Island, Tasmania (Site 1; 148.240 ^{o}E; 42.845 ^{o}S) (Fig. 1). Since gravity corers can disturb the sediment surface, a shorter (12 cm) parallel core was obtained at the same site using a KC Denmark Multi-Corer (designated 'MCS1-T6', where 'T6' refers to 'Tube 6' of the multi-corer). A 35 cm long multi-core from within Mercury Passage, west of Maria Island (Site 3; 42.550 °S, 148.014 °E) in a water depth of 68 m was also obtained (designated 'MCS3-T2'). All cores were immediately capped, sealed, labelled and transported in their original PVC coring tubes to the Australian Nuclear Science and Technology Organisation (ANSTO), Lucas Heights, Australia, where they were kept at 4 °C. GC2S, MCS1-T6, and MCS3-T6 were opened, split, scanned (using a multi-function core scanning instrument (ITRAX) with X-ray fluorescence (XRF), radiographic X-ray, optical imaging and magnetic susceptibility measurements), and subsampled for palynological and *sed*aDNA analyses in October 2018. To minimise contamination during core splitting and sampling, we wiped working benches, washed cutting knifes with 3% bleach and 70% ethanol, changed gloves immediately when contaminated with sediment, and wore appropriate PPE at all times (gloves, facemask, hairnet, disposable gown). Sampling of GC2S1 was conducted by first removing the outer ~1 cm of the working core-half and then taking subsamples by pressing sterile 15 mL centrifuge tubes ~3 cm into the sediment at 5 cm intervals (working from bottom to the top of the core at each step). Sampling of MCS1-T6 was conducted as for GC2S1 except at finer intervals of 2 cm. Mercury Passage multicore (MCS3-T2) sampling was conducted at 2 cm depth intervals in the top 8 cm and at 5 cm intervals below. All palynology and *sed*aDNA samples were immediately stored at 4 and -20 °C, respectively. Hereafter, we refer to sediment depths as 0 cm, 2 cm, etc., however, it should be noted that, due to centrifuge tubes in which samples were collected being ~1.5 cm diameter, this actually describes a sample depth interval of 0 - 1.5 cm, 2 – 3.5 cm, etc., respectively.

2.2 Sediment dating

ITRAX scanning, XRF, radiographic X-ray, optical imaging and magnetic susceptibility measurements confirmed excellent undisturbed preservation of the cores. Dating of MCS3-T2 and MCS1-T6 was based on ^{210}Pb (8 and 6 depths, respectively) and dating of and GC2S1 on both ^{210}Pb (7 depths) and ^{14}C (3 depths) measurements. A Bayesian age-depth model was

constructed for each site based on these ^{210}Pb and ^{14}C measurements using the rbacon (Blaauw et al., 2019) software in on the R platform (R Core Team, 2013) with the SHCal20 curve for radiocarbon age calibration (Hogg et al., 2020). Details on the construction of the age-depth model is provided within the Supplementary Material (Supplementary Material Fig. 1 and Table 1).

2.3 Palynological treatment and microscopy

Micropaleontological dinocyst slide preparation involved disaggregation in hydrofluoric acid (HF), density separation in a $ZnBr_2$ solution (specific gravity 2.1), sieving on 8 μm filters, and mounting in permanent mounting medium (Eukitt). One tablet of *Lycopodium clavatum* spores (Department of Geology, Lund University, Sweden, batch no. 938934, mean spores per tablet = 10,679 ± 426) was added to each sample to be used as a "tracer" as per Stockmarr (1971). Microscopy slides were counted for dinoflagellate cysts and *Lycopodinium* spores, and cysts per g^{-1} of sediment calculated by relating cysts to spore counts. Slides were analysed using a Nikon Eclipse Ci light microscope with multiple transects viewed at 200× magnification until a cyst count >100 was achieved. To improve recognition and counting of *Alexandrium* cysts, we applied primulin fluorochrome staining as developed by Yamaguchi et al. (1995) to all samples from the inshore (MCS3-T2) and shelf edge (MCS1-T6 and GC2S1) cores. While the staining protocol as originally prescribed applies to aqueous samples, both the aqueous and ethanol residues left over after palynological treatment were successfully stained. Taxonomy of the cysts was also aided by examining selected examples using a Hitachi SU-70 Scanning Electron Microscope (SEM). Nikon NIS-Elements software was used to capture light and fluorescence images during microscopy. Images were arranged for plate presentation using Microsoft Publisher. The paleontological software package TiliaIT was used for graphing of cyst profile data. A ratio was drawn between the number or dinocysts categorized as either heterotroph or autotroph per sample (Supplementary Material Table 2).

2.4 Sedimentary ancient (sedaDNA) extractions, hybridization capture and sequencing library preparations

Extractions of *sed*aDNA took place at ACAD's ultraclean ancient (GC2S1) and forensic (MCS1-T6, MCS3-T2) facilities following the recommended ancient DNA decontamination standards (Willerslev and Cooper, 2005). The extraction technique followed the new 'combined' protocol specifically developed to isolate marine eukaryote *sed*aDNA described in Armbrecht et al. (2020). This method combines a gentle EDTA incubation step to isolate fragile DNA along with a bead-beating step to extract intracellular DNA from robust spores and cyst, and further retains very small ($\geq$27 base pairs, bp) DNA fragments characteristic of ancient DNA by using in-solution silica binding and magnetic beads to size-select DNA fragments under 500 bp. *sed*aDNA extracts and metagenomic shotgun libraries were prepared from 42 sediment samples (MCS3-T2 and GC2S1) and 7 extraction blank controls following Armbrecht et al. (2021). Sequencing was undertaken using an Illumina NextSeq sequencing

platform (2 x 75 bp cycle) at the Australian Cancer Research Foundation Cancer Genomics Facility & Centre for Cancer Biology, Adelaide, Australia, and at the Garvan Institute of Medical Research, KCCG Sequencing Laboratory Kinghorn Centre for Clinical Genomics, Darlinghurst, Australia.

In order to maximise the yield of *sed*aDNA from our target dinoflagellates, we applied a hybridization-capture technique. This technique uses short RNA probes ('baits') that are designed to be complementary to any DNA sequences of interest in the DNA extract to capture and enrich these target sequences (Horn et al., 2012). In collaboration with Arbor Biosciences, USA, a 'bait set' was developed that targeted the harmful dinoflagellates *Alexandrium* groups I – IV, *Gymnodinium catenatum* and *Noctiluca scintillans* ('HABbaits1'). Details on HABbaits1 design and application to 27 selected marine *sed*aDNA extracts including protocol optimizations are provided in Armbrecht et al. (2021). A final pool of multiplexed sequencing libraries prepared from HABbaits1 was submitted for Illumina sequencing (HiSeq XTen, 2 x 150 bp cycle) to the KCCG, Darlinghurst, Australia.

Bioinformatic processing of the sequencing data (shotgun and hybridizations) followed established protocols previously described in detail in Armbrecht et al. (2020), with software versions and analytical parameters as described in Armbrecht et al. (2021). After filtering (low-complexity and duplicate reads removed), we processed each dataset *(a)* without standardising (i.e., non-rarefying) to retain the maximum number of reads, which is crucial for *sed*aDNA damage analysis (see below), and *(b)* with standardising by subsampling (i.e., rarefying) each dataset to the lowest number of reads detected in a sample, *i.e.*, 2.2 million (Mio) and 0.2 Mio for the shotgun and HABbaits1 data, respectively (using seqtk version 1.2). The latter was done to be able to assess our data semi-quantitatively. Non-rarefied and rarefied data resulting from *(a)* and *(b)*, respectively, were then continued to be processed in parallel.

After quality control (FastQC v.0.11.4, MultiQC v1.8), we used the NCBI Nucleotide database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nt.gz, downloaded November 2019) as the reference database to build a MALT index (Step 3) and aligned our sequences using MALT (version 0.4.0; semiglobal alignment) (Herbig et al., 2016). We converted all resulting .blastn to .rma6 files using the Blast2RMA tool in MEGAN (version 6_18_9, Huson et al., 2016). Subtractive filtering (*i.e.*, subtracting reads for species identified in EBCs from samples) was conducted separately for the shotgun and HABbaits1 data (see Armbrecht et al., 2021 Supplementary Material, for a comprehensive list of eukaryote contaminants), however, no Dinophyceae taxa were detected in EBCs. For each dataset (shotgun and HABbaits1), we exported the read counts for all Dinophyceae nodes (from MEGAN6 v. 18.10) for downstream analyses. Additionally, we exported read-length data (non-rarefied shotgun and HABbaits1 data) for Dinophyceae and the genera *Gymnodinium*, *Alexandrium* and *Noctiluca* (separately for each coring site) to assess whether cyst-formers preserve better than non-cyst formers (assumedly reflected in longer vs. shorter read lengths, respectively).

To test *sed*aDNA damage, we ran the 'MALTExtract' and 'Postprocessing' tools of the HOPS v0.33-2 pipeline (Hübler et al., 2020) using the same configurations as in Armbrecht et al. (2021; taxalist *'b'*, which included our three target dinoflagellate species) on shotgun and HABbaits1 *sed*aDNA data (non-rarefied, as retaining a high number of reads is critical for this step). Authenticity of the Maria Island *sed*aDNA data has been supported previously through an increasing proportion of eukaryote *sed*aDNA damage with subseafloor depth in both shotgun and HABbaits1 datasets (Armbrecht et al., 2021). Here we focus on *sed*aDNA damage analysis of the harmful dinoflagellate taxa *Alexandrium catenella*, *A. fundyense* and *A. tamarense* Group1 (hereafter grouped as *A. catenella* as these have recently all been defined as the same species, John et al., 2014), *Gymnodinium catenatum* and *Noctiluca scintillans*. To do so, we analysed the MaltExtract output, *i.e.*, reads categorized as ancient (showing damage) or default (passing stringent filtering criteria but not showing damage) for these three taxa (Hübler et al., 2020), and, based on the latter, we determined the proportion of *sed*aDNA damage per species. Also, *sed*aDNA damage profiles were generated for these three taxa using MaltExtract Interactive Plotting Application (MEx-IPA, by J. Fellows Yates; https://github.com/jfy133/MEx-IPA).

3 Results

3.1 Dinoflagellate Cysts

3.1.1 Total Dinocyst abundance

Total dinocyst abundance in the inshore core MCS3-T2 (35 cm long) was highest at the surface (3,228 cysts g^{-1} dry sediment at 5 - 6.5 cmbsf) and decreased steadily with depth (until ~30 cmbsf). A similar surface peak was observed for *Alexandrium*, *Protoceratium reticulatum*, *Protoperidinium* and *Spiniferites* taxa along with a second peak at the bottom of the core (~34 cmbsf) was only observed for the latter three taxonomic groups. At the offshore site, in MCS1-T6 (12 cm long), total cyst numbers increased with depth (5,504 g^{-1} dry sediment). In GC2S1, cyst abundance was greatest in younger sediments (3,090 g^{-1} dry sediment at 41 – 42.5 cmbsf). Summarising cyst composition as the ratio of heterotroph (mostly *Protoperidinium*) to autotroph dinoflagellate cysts (mostly *Protoceratium*, *Spiniferites*), heterotrophs contributed 40% of cysts inshore (MCS3-T2), 38% at the surface sediment at shelf edge (MCS1-T6) but decreasing to 14% at the bottom of MCS1-T6. Throughout the deeper section of the GC2S1 core the heterotroph to autotroph ratio was comparatively constant. In GC2S1 the contribution by *Protoceratium* decreased from 62% of total cysts at the surface down to 16% at the bottom at ~9,000 years old, with *Spiniferites* taking over dominance (Fig. 2).

3.1.2 Dinocyst species composition

A total of 4,279 dinoflagellate cysts comprising 32 species (28 in GC2S1, 6 in MCS1-T6, and 10 in MCS3-T2) were examined from 44 sediment samples. Most abundant offshore were the cysts of *Protoceratium reticulatum* (46 - 63% of total cysts; Fig. 3A-C), while the most abundant inshore were *Spiniferites* (including *S. bulloideus, S. hyperacanthus, S. membranaceus, S. mirabilis, S. pachydermis, S. ramosus;* combined 42% of total cysts; Fig. 3D-H) and *Protoperidinium* (*P. avellana, P. conicum, P.minutum, P. oblongum, P. subinerme, P. shanghaiense*, unindentified peridinoid "round browns"; 39% of total cysts; Fig. 3M-O). Rarer cyst species included *Impagidinium aculeatum, I. paradoxum, I. patulum, I. plicatum, I.* cf. *strialatum, I. sphaericum* (Fig. 3I) and *Nematosphaeropsis labyrinthus* (Fig. 3J) all offshore only) and *Polykrikos schwartzii* (inshore only). Typical warm-water cyst taxa such as *Lingulodinium machaerophorum* and *Tuberculodinium vancampoea* were not detected.

The microreticulate cyst of *Gymnodinium catenatum* (Fig. 3S) was only detected inshore and in very low concentrations (9 cysts in total), present in the upper part of the MCS1-T2 core with a peak of 113 cysts g^{-1} dry weight at 12 – 13.5 cmbsf (centimetre below sediment surface) and the oldest specimens (~50 years) observed at 16 cmbsf. Low concentrations of the microreticulate cysts of the smaller *Gymnodinium microreticulatum* were also detected (Fig. 3T; 3 cysts seen only, 1 each at 0 - 1.5 cmbsf, 15 – 16.5 cmbsf, and 30 – 31.5 cmbsf; Bolch and Reynolds, 2002). Detection of the bean-shaped cysts of the genus *Alexandrium* was enhanced by the use of primulin fluorescence microscopy Figs. 3 P-R). The MCS3-T2 inshore core contained *Alexandrium* cysts in 50% of sampled segments using standard light microscopy (37 cysts seen) but in 80% of samples when utilising primulin staining. Highest concentrations of 364 cysts g^{-1} dry sediment occurred in the surface sediments of MCS3-T2 and with cysts detected down to 35 cm depth (145 years old). Low concentrations of comparable cysts (23 cysts seen in total; 4 cysts g^{-1} dry sediment) were observed in the offshore GC2S1 core down to 264 cmbsf (~9,000 years old). However, none of the offshore *Alexandrium*-like cysts responded to primulin staining. Staining enhanced visibility of the archeopyle in translucent cyst taxa such as *Protoperidinium shanghaiense* (Fig. 3L) and *Spiniferites* spp. or aided detection where cysts were obscured by detrital material.

3.2 Sedimentary ancient DNA

3.2.1 Representation of Dinophyceae in shotgun data

We retrieved a total of 824,503 filtered sequences across the 42 shotgun samples, 149,892 of which were assigned to Eukaryota (18%), and 529 to Dinophyceae (0.06%). Harmful dinoflagellate taxa were detected at low abundance (19 *Alexandrium* spp., 13 *Gymnodinium* spp., and no *Noctiluca* sequences; Supplementary Material Fig. 2). Normalising the Shotgun data (i.e., rarefying/subsampling to 2.2 Mio reads per sample) reduced the number of reads assigned to Dinophyceae to a total of 148. As expected, rarefication led to the detection of less reads of harmful Dinophyceae taxa, with *Gymnodinium* only resolved to genus level in

MCS3-T2 and GC2S1, and only one *A. catenella* sequence detected in MCS3-T2 (Supplementary Material Fig. 3). In GC2S1 the read numbers showed some cyclicity throughout the core, suggesting relatively high Dinophyceae abundance (up to 10 reads per sample) between 189 and 240.5 cmbsf, and 50 - 56.5 cmbsf (Supplementary Material Fig. 3). Due to the scarcity of Dinophyceae reads in the shotgun data, we focus on presenting HABbaits1 results below.

3.2.2 Representation of Dinophyceae in HABbaits1

By applying HABbaits1 to our 27 selected *sed*aDNA extracts, a total of 32,075 sequences were retrieved and assigned to Dinophyceae, respectively. After rarefying to 0.2 Mio reads for HABbaits1, these totals were reduced to 4,456 Dinophyceae sequences. HABbaits1 provided a total of 186, 267, and 28 sequences assigned to *Alexandrium* spp., *Gymnodinium* spp., and *Noctiluca scintillans* (Fig. 4).

HABbaits1 showed that *Alexandrium* were most abundant at MCS3-T2, with the maximum number of reads (107 sequences) found in MCS3-T2 12 - 13.5 cmbsf (Fig. 4A). *A. tamarense* (not further classified) was identified at this depth (2 reads) as well as in the surface sample MCS2-T2 2 - 3.5 cmbsf. *A. catenella* was identified at MCS3-T2 6 - 7.5 cm (1 read). At GC2S1, *Alexandrium* spp. were in low abundance (<5 reads per sample), with *A. tamarense* being detected at 139 - 14.5 cmbsf (1 read, Fig. 4B). *A. catenella* were identified in GC2S1 at 75 - 76.5 (1 read) and 189 - 190.5 cmbsf (2 reads) (Fig. 4B).

A number of reads were assigned to *G. catenatum* in both the inshore and offshore core, however, given the recent introduction hypothesis, and observations that Tasmanian populations are generally estuarine-bound (McMinn et al., 1998), further inspection of these sequences was undertaken. Supplementary comparative analysis of these sequences revealed all fell within the microreticulate-group of five *G. catenatum*-like species, and the majority within 1 - 3 bp of *G. catenatum* large subunit (LSU) ribosomal rRNA genes reference sequence DQ785882 (see Supplementary Material Table 3, Supplementary Material Fig. 4,5,6,7). The *G. catenatum*-like species was identified in the surface sample of MCS3-T2 2 - 3.5 cm, alongside *G. microreticulatum* (1 read each, Fig. 4C). At GC2S1, the *G. catenatum*-like species appeared sporadically in low abundance (1 read) in the upper section of GC2S1 (above 75 – 76.5 cmbsf), while being slightly more abundant (≤6 reads) in the lower section of the core (below 169 – 170.5 cmbsf; Fig. 4D).

The application of HABbaits1 also allowed the identification of *N. scintillans* in the Maria Island sediment cores. *N. scintillans* occurred primarily in the upper section of MCS3-T2 (above 15 cmbsf), reaching its maximum abundance (9 – 10 sequences) at 6 - 7.5 and 12 - 13.5 cmbsf (Fig. 4E). This species was able to be detected in the two top samples at GC2S1 (1 sequence each, Fig. 4F). It should be noted, however, that while a low number of *G. catenatum* and *N. scintillans* sequences were detected in our deepest sample (Fig. 4D,F), this

observation should be treated with caution due to the possibility of seawater contamination of this very bottom sample during core retrieval.

3.2.3 sedaDNA damage analysis and authentication

As the shotgun dataset recovered relatively few reads from the targeted dinoflagellate taxa, we focused on analysing damage patterns in HABbaits1. HABbaits1 (non-rarefied) provided 0, 4, and 1 ancient reads for *A. catenella*, *G. catenatum*-like, and *N. scintillans* at site MCS3-T2, respectively, and 13, 95, and 11 ancient reads for *A. catenella*, *G. catenatum*-like, and *N. scintillans* at site GC2S1, respectively (Table 1). A comparatively high number of reads passed the default filtering criteria in HOPS (Table 1). Expressed in percentages, 0% and 41% of the *A. catenella* reads were classified as ancient (*i.e.*, showed clear signs of DNA damage) in MCS3-T2 and GC2S1, respectively. For *G. catenatum*-like, 4% and 27% of reads were classified as ancient in MCS3-T2 and GC2S1, respectively, and for *N. scintillans*, 0.5% and 18% were classified as ancient (Table 1). As such, reads assigned to the three target dinoflagellates showed much higher *sed*aDNA damage at the offshore site GC2S1 relative to the inshore site MCS3-T2. Due to the scarcity of reads categorized as ancient, few *sed*aDNA profiles could be generated, especially for the inshore site MCS3-T2, however, all profiles generated for the three target dinoflagellates are included in Supplementary Material Fig. 8 for completeness.

3.2.4 Sequence length analysis of cyst vs. non-cyst formers

Slight differences in *Alexandrium*, *Gymnodinium*, and *Noctiluca* read lengths were found when compared to overall Dinophyceae read lengths in the shotgun and HABbaits1 datasets. However, due to very low read numbers recovered by the shotgun data, and high standard deviations of average fragment lengths per taxon, we do not consider these statistically robust, thus provide this data with the Supplementary Material (Supplementary Material Fig. 9).

4 Discussion

This study presents the first marine sediment core analysis of dinoflagellate cyst assemblages off the Tasmanian east coast and defines a clear demarcation between long-term dinoflagellate cyst species in subtropical New South Wales waters (McMinn, 1992) and temperate Tasmanian waters. Typical warm-water cyst taxa such as *Lingulodinium machaerophorum* and *Tuberculodinium vancampoea*, common in New South Wales, were notably absent off Maria Island, and reflect limitations in the southward penetration of the East Australian Current into Tasmanian shelf waters throughout the past ~9,000 years. The most prominent inshore cyst taxa were *Spiniferites* spp. whilst cysts of *Protoceratium reticulatum* dominated offshore. *Protoperidinium* displayed episodic peaks inshore, but were more consistently present offshore. Through a combined approach of palynology and

*sed*aDNA analysis, this study provides the first evidence of the presence and changing abundance of the three harmful dinoflagellates *A. catenella*, *G. catenatum*-like, and the soft-bodied non-cyst-former, *N. scintillans* in Australian waters covering a ~9,000-year time-period.

4.1 HAB species presence around Maria Island, Tasmania, over the last 9,000 years

Alexandrium

Both palynology and *sed*aDNA showed that the *A. tamarense* species complex has been present inshore and offshore of Maria Island since ~145 and at least ~9,000 years, respectively. *Alexandrium* can be a prolific cyst producer (by approximately 40% of vegetative cells; Anderson et al., 2014). Inshore, *Alexandrium* cysts could be clearly identified via primulin staining, while surprisingly offshore none of the *Alexandrium*-like cysts responded to the same staining. This may suggest degradation of the cellulose cyst wall at sediment ages exceeding ~145 years. Although we consider the following less likely due to detailed inspection by microscopy, the lack of staining could also mean that these were non-*Alexandrium* cysts (Yamaguchi et al., 1995). However, *sed*aDNA (HABbaits1) implied that the genus *Alexandrium* became abundant in inshore waters in recent years (~15 years ago to present), and also offshore at around 2,300, ~6,000, and prior to ~7,200 years ago. The offshore *Alexandrium sed*aDNA peak at 2,300 years ago matches a putative cyst peak (supporting the identification of these cysts as *Alexandrium* despite non-staining). *sed*aDNA (HABbaits1) identified *Alexandrium* spp. at nearly all sampled depths of GC2S1, including the toxic *A. catenella* at ~3,500 and ~7,200 years ago (Fig. 4B). This observation confirms the previous suggestion that this species has been endemic in the Tasmanian region before being stimulated by climate changed-induced environmental conditions (Condie et al., 2019). The HABbaits1 enriched *sed*aDNA was unable to discriminate between *Alexandrium* genotypes 1 (*catenella*), 4 (*pacificum*) and 5 (*australiense*) through the core because the fragments retrieved mapped to somewhat more conserved gene regions including the rRNA gene. Increased starting material and HABbaits1 enrichment, combined with increased sequencing depth would provide higher probability of recovering *sed*aDNA fragments from the most informative regions of the rRNA genes such the D1-D2 regions of the LSU, and the rRNA ITS-regions successfully targeted in these species for detection using qPCR (e.g., Ruvindy et al., 2018).

Gymnodinium

Both palynology and *sed*aDNA identified *G. catenatum* at the inshore site at ~15 (*sed*aDNA) and ~30 years ago (palynology) and, to a lesser degree in recent sediments ~2 years ago (palynology and HABbaits1). A presence 30 years ago (~1990) corresponds to a period when *G. catenatum* represented approx. 9% of resting cysts in surface sediments of Spring Bay (Bolch and Hallegraeff 1990). No *G. catenatum* cysts were detected in the offshore core using palynology, but it is noted that *G. catenatum* is a very poor cyst producer (1 - 2% of vegetative cells; Blackburn et al., 2001). *sed*aDNA identified *G. catenatum*-like sequences sporadically throughout the entire length of GC2S1, with slightly higher representation at greater depths (~6,700 - 9,000 years ago). This was unexpected as, based on duplicate

sediment cyst core studies in the neighbouring Huon River (100 km distant), *G. catenatum* is thought to have been introduced to Tasmania via ship's ballast water in the 1970s (McMinn et al., 1998).

The reliability of species assignments of *Gymnodinium* spp. from rRNA genes (internally transcribed spacer (ITS), small subunit (SSU) and large subunit (LSU) rRNA) using a synthetic dataset showed that 18S rRNA (SSU) sequences of *G. catenatum* and *G. microreticulatum* can only be confidently assigned to order-level (*e.g.*, Gymnodiniales), especially when sequences are short as were our *sed*aDNA sequences (~56 bp on average in the shotgun data) (see Supplementary Material Table 3, Supplementary Material Fig. 4). However, in this study we recovered LSU rRNA gene sequences, which were (in contrast to SSU) assignable below genus level. All *G. catenatum*-related *sed*aDNA LSU-rRNA gene sequences (44 – 129 bp) mapped downstream of the LSU D1-D3 region and were confirmed to be most similar to *G. catenatum* but not an exact match (Supplementary Material Fig. 5,6,7). The latter was determined by mapping the *sed*aDNA sequences to publicly available *Gymnodinium* LSU reference sequences (which has low taxon coverage as only three reference sequences are available, *G. catenatum*, *G. aureolum*, *G. impudicum*), as well as to newly, in-house generated sequences of the LSU D3-D10 region (~2,000 bp) of *G. microreticulatum* and *G. nolleri* (Supplementary Material Fig. 5,6,7). The *G. catenatum* LSU reference sequence DQ785882 which the *sed*aDNA fragments matched most closely is from a cultured Korean *G. catenatum* isolate, and the small (1 – 3 bp) differences may reflect sequence variation between Australian and other populations (e.g., Bolch and De Salas, 2007) or perhaps the existence of related but as yet undescribed or unreported cryptic species. Sequence variation among dinoflagellates across the D3-D10 region is also considerably lower than the hypervariable regions between D1 and D2. When combined with the low taxon coverage, we cannot conclusively determine whether these sequences represent *G. catenatum* or another related microreticulate group species. A recent introduction via ballast water, superimposed upon an indigenous cryptic population remains a possible interpretation. Additional studies with increased sample and/or sequencing depth and improved coverage of full length rRNA genes from gymnodinoid taxa are necessary more firmly establish the most probable explanation for our data.

Noctiluca

HABbaits1 revealed the presence of *N. scintillans* inshore of Maria Island over the last ~30 years, as well as traces of this dinoflagellate in shallow sediments offshore (0 – 1.5 and 5 – 6.5 cmbsf; <100 years old). Inshore, the highest abundances of *N. scintillans* were detected in relatively recent sediments (6 – 7.5 and 12 – 13.5 cmbsf), indicating bloom phases of this species occurred within the last 7 – 15 years, corroborating the observational record. *N. scintillans* was first detected in Tasmania in 1994 and thought to be driven by increased southward extension of the East Australian Current. Since then, blooms of this dinoflagellate have increased in both frequency and intensity (Hallegraeff et al., 2019). To our knowledge, this is the first time that *N. scintillans sed*aDNA has been reported from a coastal ecosystem into which it has been introduced, demonstrating the sensitivity of the HABbaits1 approach

for detecting plankton community change of fragile non-fossilizing species from the marine sediment record. *N. scintillans* was identified in the deepest sample at GC2S1, however, our HOPS analysis showed that *sed*aDNA damage in this sample was very low (1 ancient vs. 32 default reads in the non-subsampled HABbaits1 data), so we cannot exclude the possibility these are modern sequences originating from seawater contamination during coring.

4.2 Reconstructing paleo-blooms

The abundance of *Alexandrium*, *Gymnodinium*, and *Noctiluca* through time were assessed using subsampled shotgun and HABbaits1 datasets. The shotgun data showed variations in the total abundance of Dinophyceae at the offshore site, and periods of increased abundance at ~2,300 and ~7,200 – 9,000 years ago largely contributed by increased *Gymnodinium* spp. (Supplementary Material Fig. 3). However, differences in gene copy number amongst species can influence their over-/under-representation in sedaDNA therefore the data should be viewed as semi-quantitative. To investigate whether individual HAB-species might have undergone bloom-phases, HABbaits1 data was examined separately for each of the three dinoflagellates of interest. By doing so, it was determined that high abundances of *Alexandrium* spp. at ~15 years ago, indicate a bloom phase (inshore). *G. catenatum*-like read numbers were elevated between ~ 6,700 – 9,000 years ago, but as indicated above this species assignment is unclear. Blooming of *N. scintillans* in recent years, particularly inshore, is supported through elevated abundances of reads of this species between 17 - 15 years ago. Similar 'appear and disappear' HAB patterns have started to emerge from both palynological and sediment environmental DNA studies in the Northern Hemisphere. For example, *Gymnodinium catenatum* first became a problem in Spain and Portugal in 1976, but cyst records date back to the late 1800s, 75 years before the first human PSP poisonings occurred (Ribeiro et al., 2012). *Alexandrium minutum* has been associated with PSP problems in the Bay of Brest, France only since 2012, but environmental DNA studies of sediment cores demonstrated its presence in the area since the late 1800s (Klouch et al., 2018).

4.3 Characteristics of harmful dinoflagellate sedaDNA preserved in coastal marine sediments off Eastern Tasmania and implications for long-term preservation

Our *sed*aDNA approach allowed the assessment of authenticity for sequences recovered for *A. catenella*, *G. catenatum*-like, and *N. scintillans*. The % *sed*aDNA damage was low for sequences belonging to the three target taxa at MCS3-T2 (up to 4% for *G. catenatum*-like), however, this was expected and in line with the finding that eukaryote *sed*aDNA damage is generally very low in the upper ~35 cm of sediment at this site (Armbrecht et al., 2021). Offshore, the increased proportion of *sed*aDNA damage observed (up to 41% for *A. catenella*) indicate that the *A. catenella*, *G. catenatum*-like and *N. scintillans* sequences are authentic, with the exception of the bottom-core sample where contamination by seawater cannot be ruled out.

The advantage of combining palynological and *sed*aDNA records to investigate the long-term presence of harmful dinoflagellates becomes clear when soft-bodied, non-cyst-forming species are considered. Our *Noctiluca sed*aDNA evidence matches the observational records, and the reduced relative abundance offshore is consistent with *N. scintillans* blooms being predominantly coastal (Hallegraeff et al., 2019). However, the presence of *N. scintillans sed*aDNA in recent sediments at the offshore site confirmed that its DNA has been preserved at the seafloor after sinking through a 140 m water column. Future research into the mode of downward transport (*e.g.*, sinking after dying, or transport inside copepod faecal pellets) might provide further insights into this new finding. While determined from relatively low number of reads (60), *N. scintillans sed*aDNA shows signs of damage, especially at the offshore site, indicating that the DNA of this soft-bodied dinoflagellate does not preserve well for long and may pose limitations to its analysis using *sed*aDNA records. Future investigations could target sediment cores from Sydney, where this species has been present closer to shore for longer and rate of damage of *N. scintillans sed*aDNA might be estimated over longer timescales.

The microfossil and *sed*aDNA data presented here both support the suggestion that *A. catenella* has been present in Tasmania throughout the last ~9,000 years and that recent bloom events are associated with alterations in oceanographic conditions (Condie et al., 2019). Hybridization capture *sed*aDNA confirmed the presence of *Alexandrium* offshore where unambiguous identification by microscopy was limited due to the lack of a primulin staining response by the cysts. It is noteworthy that toxic *Alexandrium catenella* blooms may have occurred around 3,500 years ago (supported in this study by *sed*aDNA), as this falls into the time period when Tasmanian aboriginals reportedly stopped (or, at least limited) to consume scaled fish (~3,000 - 4,000 BP; Jones, 1978, Taylor, 2007), however, a relationship between these two events is entirely speculative.

Detecting *G. catenatum sed*aDNA sequences in GC2S1 samples of up to 9,000 years in age conflicts with a well-established recent introduction hypothesis. While the *sed*aDNA fragment assignment process used in this study proved to be robust, the limited availability of gymnodinoid reference sequences covering the full-length LSU rRNA gene operon sequences means conclusive identification of the *G. catenatum*-like species in *sed*aDNA was not possible. This has two significant consequences for the use of *sed*aDNA to infer the past plankton community structures over longer timescales. First, it demonstrates the importance of closely investigating the source, characteristics, and authenticity of the typically very short *sed*aDNA fragments before drawing conclusions; second, the urgent need for more complete reference sequences for many species of interest (including *Gymnodinium*) if robust species-level resolution is anticipated.

5 Conclusions

Our study provides new insights into the long-term presence and prevalence cycles of three HAB species in the Tasmanian region, including preliminary clues to their blooming phases. Both palynological and *sed*aDNA analyses confirmed the presence of *Alexandrium* over thousands of years. Strikingly, *Alexandrium* cysts from inshore sediments responded to primulin fluorescence staining, but older offshore *Alexandrium* cysts did not, the significance of which is unclear. The detection of cysts of *Gymnodinium catenatum* in 50-year-old inshore sediments support the body of evidence of a ballast water introduction into Tasmania, previously postulated from shorter (25 to 80 cm long, 100 - 200-year-old) Huon River sediment cores (McMinn et al., 1998). However, the occurrence of *G. catenatum*-like *sed*aDNA throughout the offshore core suggests a possible similar long-time history (thousands of years) for a *G. catenatum* related species in Tasmanian waters, requiring further research to confirm the authenticity and identity of these sequences. From *sed*aDNA hybridization capture data we confirm that the range expansion of the non-cyst forming, soft-walled *Noctiluca* to Tasmanian waters represents an unprecedented change to this regional marine ecosystem since the 1990s. Application of similar methods to the putative range expansion of green *Noctiluca* (with green algal symbionts) into the Arabian Sea (Gomes et al., 2014) would be of considerable interest.

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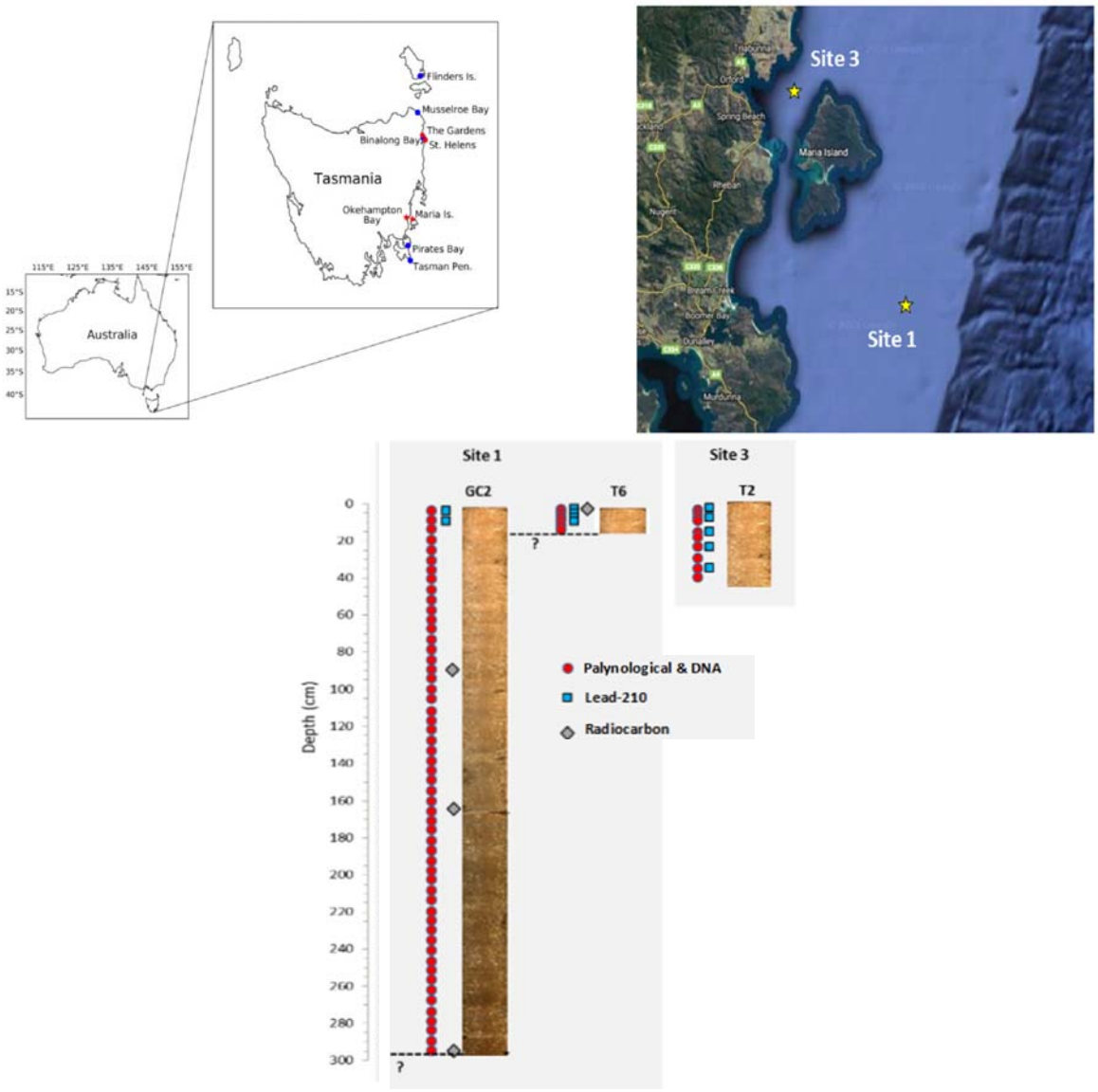

Figure 1: Sediment coring sites near Maria Island, Tasmania, Australia. Overview of coring locations offshore (Gravity Core Site 1, GC2S1, and Multi-Core Site 1 Tube 6, MCS1-T6, surface section only) and inshore Maria Island in the Mercury Passage (Multi-Core Site 3 Tube 2, MCS3-T2). Red dots indicate palynology and *sed*aDNA sampling depths, blue rectangles indicate Lead-210 (Pb^{210}) dating sampling depths, and grey diamonds indicate radiocarbon (^{14}C) dating sampling depths.

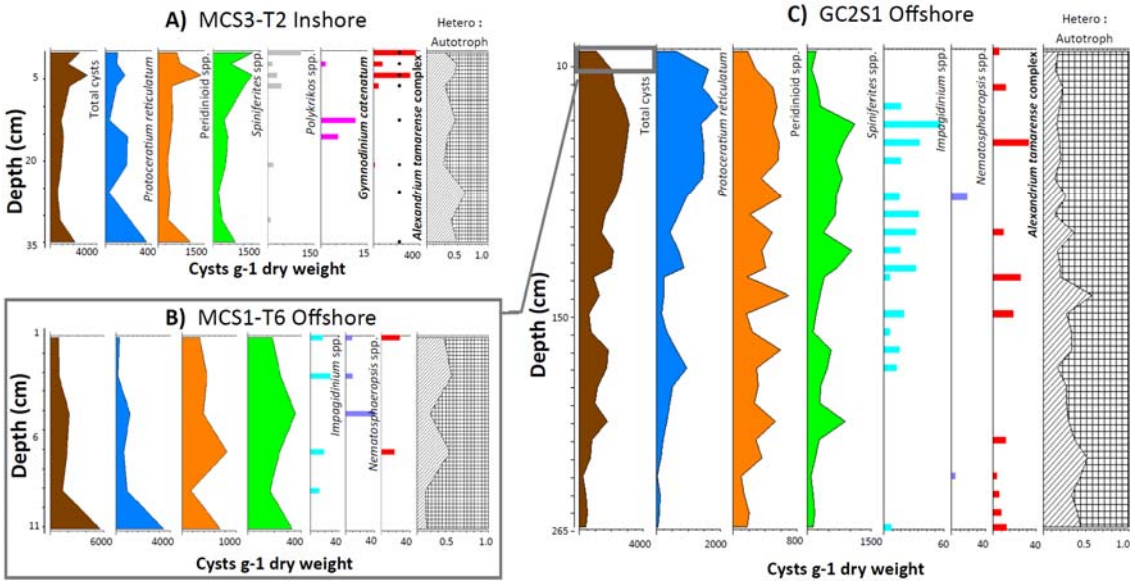

Figure 2: Dinoflagellate cyst abundance at MCS3-T2 (inshore), and MCS1-T6 and GC2S1 (offshore) cores determined from palynological analyses. Most abundant were cysts of *Protoceratium reticulatum, Spiniferites* and *Protoperidinium* species. *Impagidinium* and *Nematosphaeropsis* occurred offshore only, and *Polykrikos* and *Gymnodinium catenatum* were detected inshore only. *Alexandrium*-like cysts occurred both inshore and offshore. Cyst composition is also summarized as the ratio of heterotroph (mostly *Protoperidinium*) to autotroph taxa (mostly *Protoceratium, Spiniferites*).

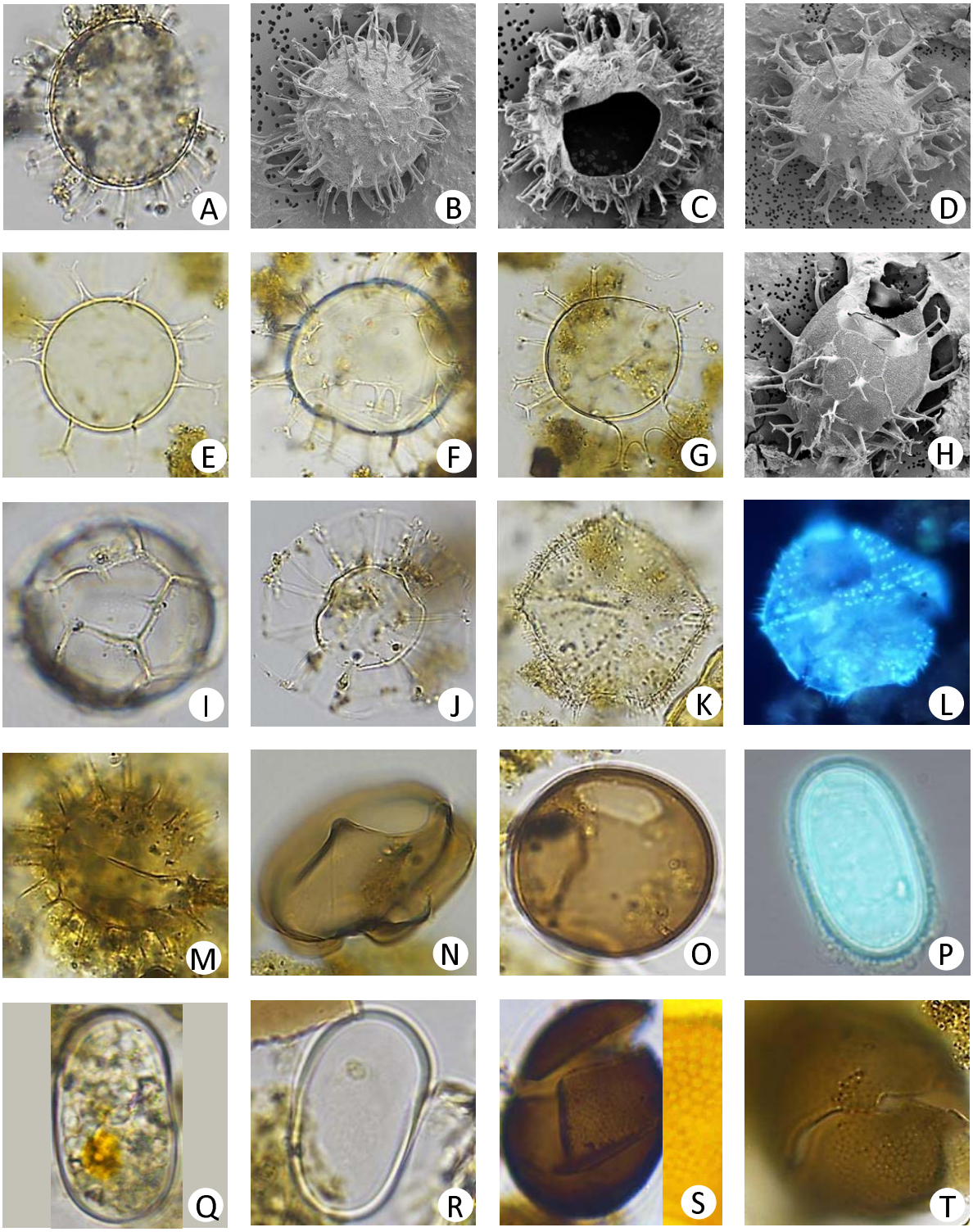

Figure 3: Maria Island dinocysts. A,B,C. Cysts of *Protoceratium reticulatum* with long processes (diameter 35µm), **D**,**E.***Spiniferites bulloideus* (diameter 40µm), **F.** *Spiniferites hyperacanthus* (diameter 55µm); **G:** *Spiniferites mirabilis* (diameter 50µm), **H:** *Spiniferites ramosus* main body diameter 55µm),, **I:** *Impagidinium sphaericum* (diameter 45µm., **J:** *Nematosphaeropsis labyrinthus* (main body diameter 30 µm); **K,L:** *Protoperidinium*

shanghaiense (length70 µm), L. after primulin staining; . **M:** *Protoperidinium conicum* (main body diameter 55µm), **N:** *Protoperidinium subinerme* (diameter 55 µm),), **O:** *Protoperidinium avellana* (diameter 40µm), **P,Q,R.** *Alexandrium tamarense* complex (length 35µm), P . after primulin staining; T2 1cm; Q. live cyst with contents; R. T2 1cm ; **S:** *Gymnodinium catenatum* (diameter 50µm). + Detail of microreticulate ornamentation**; T:** *Gymnodinium microreticulatum* (diameter 25µm)

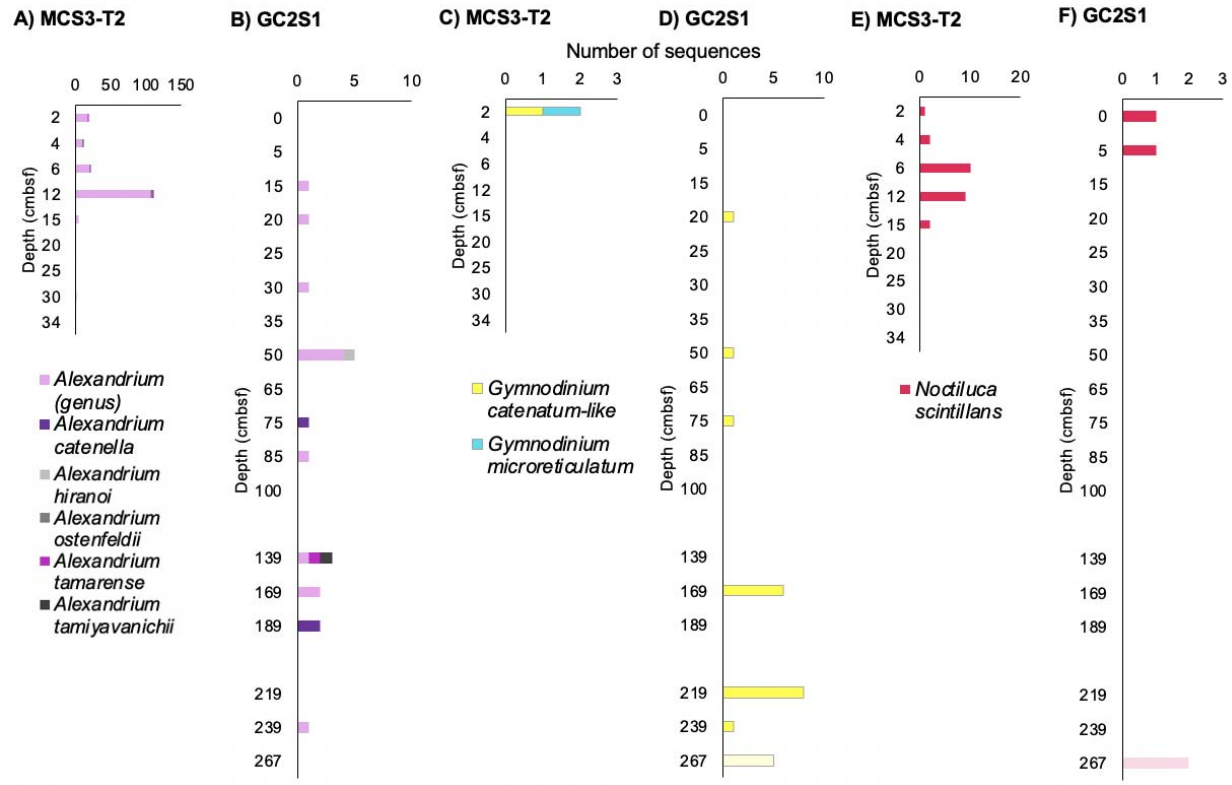

Figure 4: Abundance of *Alexandrium* spp., *Gymnodinium catenatum*-like species and *Noctiluca scintillans* as determined by *sed*aDNA inshore and offshore Maria Island. A-F) Normalized (rarefied) data derived after application of hybridization-capture using HABbaits1. A,C,E) Inshore site MCS3-T2. B,D,F) Offshore site GC2S1. D,F) Light colouring of bottom sample read counts indicates that contamination with modern sequences cannot be excluded.

868 **Table 1: *sed*aDNA damage of reads assigned to the harmful dinoflagellate taxa *A.***
869 ***catenella*, *G. catenatum*-like and *N. scintillans*.** The total number and proportion of reads
870 classified into ancient and default via HOPS *sed*aDNA damage analysis (based on HABbaits1
871 data). The proportion of ancient reads is a measure of '% *sed*aDNA damage' for each of the
872 three species (in italics).

Total	**GC2S1**		**MCS3-T2**	
Taxa	**Ancient**	**Default**	**Ancient**	**Default**
A. catenella	13	19	0	6
G. catenatum-like	95	252	4	97
N. scintillans	11	49	1	213
Proportion (%)	**GC2S1**		**MCS3-T2**	
Taxa	**Ancient**	**Default**	**Ancient**	**Default**
A. catenella	*41*	59	*0*	100
G. catenatum-like	*27*	73	*4*	96
N. scintillans	*18*	82	*0.5*	99.5

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