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RESEARCH ARTICLE

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Not dead yet: Diatom resting spores can survive in nature for several millennia

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Abstract

Premise: Understanding the adaptive capacities of species over long timescales lies in examining the revived recent and millennia-old resting spores buried in sediments. We show for the first time the revival, viability, and germination rate of resting spores of the diatom *Chaetoceros* deposited in sub-seafloor sediments from three ages (recent: 0 to 80 years; ancient: ~1250 (Medieval Climate Anomaly) and ~6600 (Holocene Thermal Maximum) calendar year before present.

Methods: Recent and ancient *Chaetoceros* spores were revived to examine their viability and germination rate. Light and scanning electron microscopy and Sanger sequencing was done to identify the species.

Results: We show that ~6600 cal. year BP old *Chaetoceros* resting spores are still viable and that the vegetative reproduction in recent and ancient resting spores varies. The time taken to germinate is three hours to 2 to 3 days in both recent and ancient spores, but the germination rate of the spores decreased with increasing age. The germination rate of the recent spores was ~41% while that of the ancient spores were ~31% and ~12% for the ~1250 and ~6600 cal. year BP old resting spores, respectively. Based on the morphology of the germinated vegetative cells we identified the species as *Chaetoceros muelleri* var. *subsalsum*. Sanger sequences of nuclear and chloroplast markers identified the species as *Chaetoceros muelleri*.

Conclusions: We identify a unique model system, *Chaetoceros muelleri* var. *subsalsum* and show that recent and ancient resting spores of the species buried in sediments in the Baltic Sea can be revived and used for long-term evolutionary studies.

KEYWORDS

Baltic Sea, *Chaetoceros muelleri* var. *subsalsum*, germination rate, resting spore concentration, resurrection ecology

Phytoplankton forms the basis of the marine food web and hence, if we understand how phytoplankton will respond to environmental and climate change, we will have a better chance to understand ecosystem change. Changes in species' abundance and significant loss of biodiversity in the marine environment have occurred and the numbers of planktic, resistant, toxic, and introduced species have increased due to nutrient enrichment resulting in eutrophication and hypoxia, and also metal pollution and acidification (Yasuhara et al., 2012). The lack of a good model system to understand how the adaptive responses of species will be affected by anthropogenic perturbations over long timescales has emerged as a major challenge (Schlüter et al., 2014; Hofman et al., 2015). Our current inability to do so hampers our understanding of how the future ocean will function.

Resurrection ecologists have long recognized sediments as sources of viable propagules ("seed or egg or resting spore banks") for studying ecological and evolutionary responses (Cáceres and Hairston, 1998). Resting stages in the sediment are of ecological and paleoecological importance as they can

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be revived when exposed to suitable environmental conditions and used as a source of genetic material for microevolutionary studies (Ellegaard and Ribeiro, 2017). Hence, dormant propagules in sediments are natural archives from which adaptive trajectories of populations could be traced through extended time periods. Currently, only diatom spores have been revived from century-old sediments (Härnström et al., 2011), and studies have shown that the survival periods of several marine dinoflagellate resting stages range from several months to 100 years (Miyazono et al., 2012). Previous studies on *Daphnia* and *Sphagnum* have resurrected up to 700 years old ancient eggs (ephippia) and spores (Bu et al., 2017; Yousey et al., 2018). Thus, these resting spores can provide us with a good study system as we currently lack a model system to study the changes occurring over longer evolutionary timescales. One potential key to understanding the adaptive capacities of species over evolutionary time lies in examining the recent and millennia-old resting spores buried in the sediments.

Resting spore formation in diatoms is an effective strategy to survive periods of stress and has enabled diatoms to withstand events of mass extinction during the end of Cretaceous period (Kitchell et al., 1986). Climate, nutrient concentration, and anthropogenic disturbances can influence the distribution, composition, abundance, and adaptation of diatom species (Crosta et al., 1997) thus rendering them a useful probe to study environment induced adaptations over long timescales (Orsini et al., 2013; Burge et al., 2017).

In this context, the Baltic Sea is an ideal ecosystem for conducting long-term evolutionary studies as diatom resting spores are found throughout the brackish sediment stratigraphy in the open Baltic Sea. The history of the Baltic Sea begins after the last glacial maximum about 22000 cal. yr BP, when deglaciation of the Fennoscandian Ice Sheet began (Stroeven et al., 2016). During and following deglaciation the Baltic Basin went through several different freshwater to brackish water stages due to isostatic rebound and eustatic sea level changes during a geologically and evolutionarily short period of time (Andrén et al., 2011; Snoeijs-Leijonmalm and Andrén, 2017). These stages are described as Baltic Ice Lake (freshwater—ca. 16000 to 11700 cal. yr BP), Yoldia Sea (freshwater with short brackish phase—11700 to 10700 cal. yr BP), Ancylus Lake (freshwater—10700 to ca. 9800 cal. yr BP), Initial Littorina Sea (slightly brackish water—ca. 9800 to ca. 8500 cal yr BP), Littorina Sea (brackish water—ca. 8500 to 3000 cal. yr BP), Post Littorina Sea (3000 cal. yr BP and transition to present day Baltic Sea) and are reflected in the lithology as different lithological units (Andrén et al., 2011; Weckström et al., 2017). The Littorina Sea (~8500 to 3000 cal. yr BP) reached a maximum surface water salinity of 12 to 13 PSU in the Baltic Proper (that area of the Baltic Sea, from Åland Sea to the Danish sounds), compared to present day 5 to 7.5 PSU (Widerlund and Andersson, 2011). The higher salinity coincides with the Holocene Thermal Maximum (HTM), with warm and dry climate dated to ~8000 to 5000 cal. yr BP (Seppä et al., 2009). At present, the Baltic Sea consists of a mixture of marine North Sea water and freshwater runoff, which results in a long salinity gradient ranging from the transition zone in Kattegatt (18–26 PSU), to the major part of the Baltic Sea (5–8 PSU) and the almost freshwater Bothnian Bay (2–4 PSU) (Leppäranta and Myrberg, 2009; Snoeijs-Leijonmalm and Andrén, 2017). The Baltic Sea has strong horizontal (salinity, temperature) and vertical (also including an oxygen gradient) environmental gradients (Snoeijs-Leijonmalm and Andrén, 2017). The vertical salinity gradient results in stratification, which together with ongoing eutrophication causes large areas with hypoxic and anoxic (oxygen concentration <2 and <0 mg L-1) bottom waters (Carstensen et al., 2014).

Diatoms are one of the dominant types of phytoplankton in the Baltic Sea ecosystem, playing a major role in pelagic primary production (Andersson et al., 2017). *Chaetoceros* is an abundant and diverse marine planktic diatom genus both globally and in the Baltic Sea and plays a major role in marine primary production (Malviya et al., 2016). Studies in the Baltic Sea area show that the present distribution of *Chaetoceros* species correlates with the salinity gradient resulting in higher diversity in the more marine Kattegatt and Danish straits and lower diversity in the brackish central Baltic Proper and in the nearly freshwater Bothnian Bay (Hällfors, 2004). *Chaetoceros* is a genus that produces resting spores. Due to its dissolution-resistance and a high sedimentation rate (which result in quick burial of the spores) in the Baltic basin, *Chaetoceros* resting spores are found throughout the sediment stratigraphy from when marine water first entered the Baltic Proper (during the Littorina Sea, ~8500 years ago) until the present (Andrén et al., 2000; Witak et al., 2011). In the open Baltic Proper, high *Chaetoceros* resting spore concentrations are found in the laminated, hypoxic sediments deposited during three time periods: (1) the last century; (2) the Medieval Climate Anomaly (MCA, ~1000 to 700 cal. yr BP; Mann et al., 2009); and (3) the HTM (E. Andrén et al., 2000). The abundances of resting spores in sediments from the Baltic Basin are attributed to high primary production and eutrophication (E. Andrén et al., 1999, 2000) and also coincides with high temperatures and higher surface water salinities (Zillén et al., 2008; E. Andrén et al., 2020).

To systematically study the long-term effects of natural variation in climate happening over long time scales on the ecosystem affected by multiple stressors like eutrophication and global warming during the last century, we need a model organism whose resting spores have accumulated and have been preserved in the sediment. The overall objective of the present study is to determine if *Chaetoceros* resting spores preserved in Baltic Sea sediments have the potential to be a good model system to study the impact of natural variation in climate and anthropogenic perturbations. The specific aims are to: (1) examine the resting spore concentration from different time periods (recent years, MCA, HTM); (2) determine the age of the sediments with high resting-spore concentrations by radiocarbon dating; (3) examine if the resting spores from different ages with

high resting-spore concentrations can be revived by germinating them; (4) identify germinated *Chaetoceros* spores to species' level (since many spores have identical morphology in comparison to germinated living cells) by using light and scanning electron microscopy; and (5) identify the germinated *Chaetoceros* species based on DNA sequences.

MATERIALS AND METHODS

Sampling

The Integrated Ocean Drilling Program (IODP) Expedition 347 drilled cores in the Baltic Proper from R/V *Greatship Manisha* from September to November 2013. The M0063 site (58°37.32′N, 18°15.24′E) in the Landsort Deep, the deepest part of the Baltic Proper at a water depth of 437 m, had cores drilled during this expedition (Figure 1). Five parallel holes (M0063A to M0063E) were drilled, using an advanced piston corer with perfluorocarbon added in the liner fluid to trace possible contamination, down to a diamicton (till) was reached at about 92 meters composite depth (mcd) (T. Andrén et al., 2015). The sediments were stored in the dark in a cold room at 4°C. All Expedition 347 cores were split and sub-sampled during the onshore science party at the IODP Core Repository in Bremen, Germany, January to February 2014. Subsamples from hole D were used for resting spore concentration analyses, since this hole was used for all biostratigraphic analyses of site M0063 carried out by the science group. To be able to carry

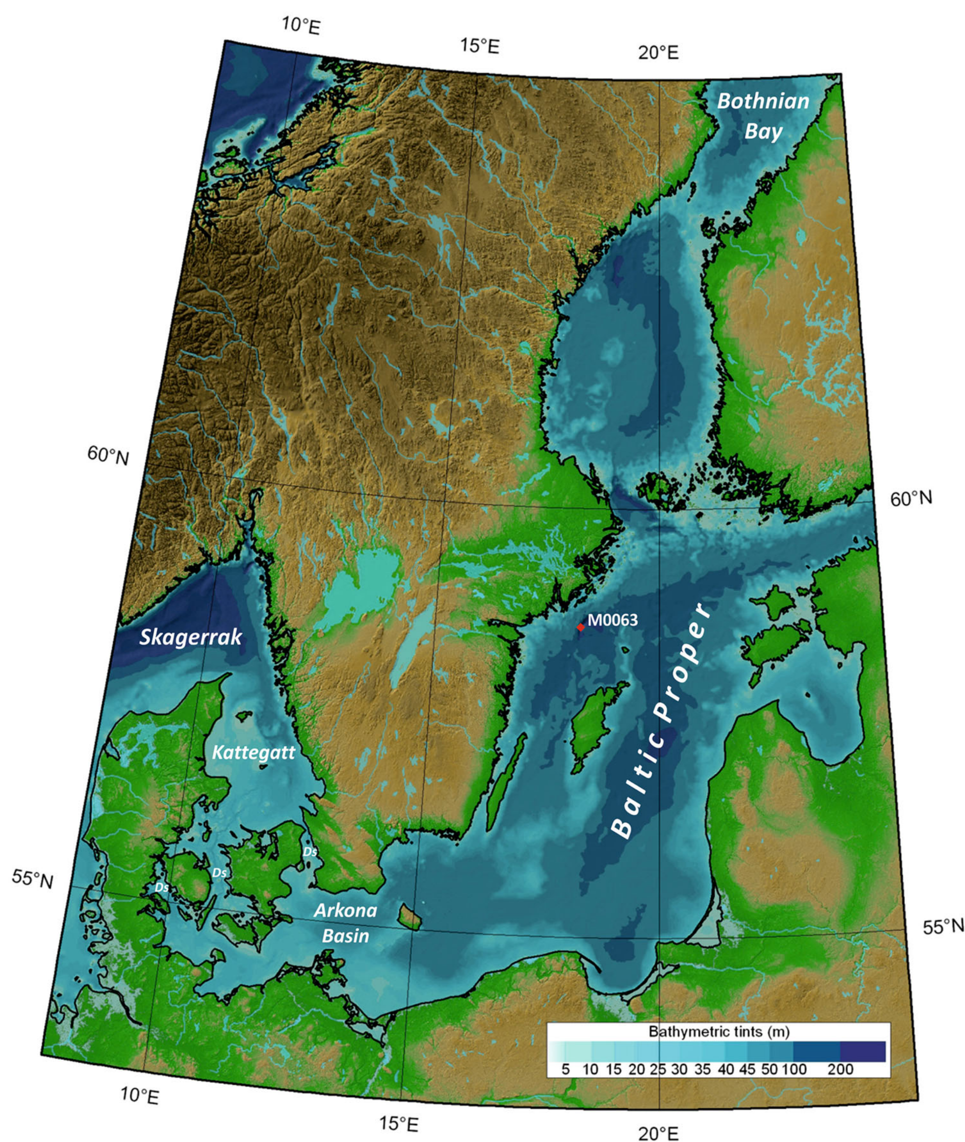

FIGURE 1 Map showing the position of the investigated IODP Expedition 347 site M0063 in the Landsort Deep (58°37.32′N, 18°15.24′E) where a ca. 116 m long sediment core was drilled at a water depth of 437 m (T. Andrén et al., 2015). Baltic Sea has a long salinity gradient in the surface waters ranging from ~12 to 30 PSU in Kattegatt, ~10 to 23 PSU in Danish straits (Ds), ~8 to 11 PSU in Arkona Basin, 5 to 7.5 PSU in Baltic Proper, and ~2 to 4 PSU in Bothnian Bay (Andersen et al., 2017)

out the revival experiments more and freshly subsampled sediment material was needed, a sample request was submitted in 2015 to the curators of the IODP Core Repository in Bremen. As limited amount of sediment remained in hole D, we received subsamples also from the parallel hole C. To collect the topmost unconsolidated recent sediments, the drilling site was revisited in June of 2014 with M/S *Fyrbyggaren* and the topmost unconsolidated sediments were sampled with a 1-m gravity corer. Two cores 87 cm (named M0063H at N58°37.34′, E18°15.29′) and 63 cm (named M0063I at N58°37.35′, E18°15.25′), respectively were retrieved and immediately subsampled in 1-cm slices and stored in the dark in a cold room at 4°C.

Lithology and sample selection

The lithology at site M0063 is divided into seven lithostratigraphic units very briefly described as: below 92 mcd diamicton; 92 to 32 mcd laminated clays on cm-scale which gradually change to mixed clays in the upper part; 32 to 26 mcd homogeneous clays with sulphide banding; and 26 to 0 mcd organic rich clays with somewhat pronounced laminae on mm-scale (T. Andrén et al., 2015).

Based on the results from the onshore science group (T. Andrén et al., 2015), samples from sediment sections of three different ages (the last century (M0063I—0 to 63 cm), MCA (M0063C—5.42 to 6.32 mcd), and the HTM (M0063D—21.63 to 26.05 mcd) with anticipated high spore abundance were selected to trace the genetic diversity and the evolutionary changes in the *Chaetoceros* populations.

Dating and age modelling

Bulk sediment samples from the two selected time intervals corresponding to the MCA (hole C) and the HTM (hole D) were dated by radiocarbon accelerator mass spectrometry (AMS) at Beta Analytic Inc. in Miami, Florida, USA.

In order to assign a calendar year age to each level analyzed, an age-depth modeling was performed for the sequences analyzed in both cores using the software BACON version 2.5.1 (Blaauw and Christen, 2011) using the *IntCal20* calibration dataset (Reimer et al., 2020) with a mean and standard deviation of 900 and 500 ^{14}C years respectively (Obrochta et al., 2017). The sediment surface is assumed to be modern (i.e., 2013, the year of the core drilling).

The youngest sediment (the two short gravity cores M0063H and M0063I), was dated using stratigraphic time markers (Moros et al., 2017). In order to identify stratigraphic time markers, mercury (Hg) and artificial radionuclide (^{137}Cs and ^{241}Am) measurements on M0063H have been performed. Hg was measured on sample weights of 20 to 100 mg using a DMA-80 analyzer from Milestone Srl (Sorisole, Italy) (for details, see Leipe et al., 2013). Analyses of the artificial radionuclides ^{137}Cs and ^{241}Am were carried out by gamma spectrometry with the Canberra well detector GCW4021-7500SL-RDC-6-ULB (for details, see Moros et al., 2017). Core correlation of M0063H and M0063I is based on characteristic features of the mercury downcore profiles. Hg and ^{137}Cs were normalized to bulk (total) organic carbon (TOC) in order to eliminate the dilution effect of the massively occurring manganese-carbonate layers. Total carbon content was measured using an EA 1110 CHN analyser from CE Instruments (Lancashire, UK), total inorganic carbon (carbonates) content with a Multi EA-2000 elemental analyser from Analytik Jena (Jena, Germany), and TOC was calculated as the difference between them. Analyses of carbon, mercury, and artificial radionuclides were carried out at the Leibniz Institute for Baltic Sea Research in Warnemüende, Germany.

Resting spore concentration

To calculate the concentration of resting spores in the upper 30 m (mcd) from hole M0063D, sediments were freeze-dried, and a known weight of sediment (on average ~0.1 g) was subsampled. Cleaning of diatoms was performed according to standard procedures (Battarbee 1986). Microspheres were added in the last step to allow for the calculation of resting spore concentrations before mounting for permanent slides in Naphrax™ (Brunel Microscopes Ltd, Wiltshire, UK) (refraction index n_D=1.73) (Battarbee and Kneen, 1982). A total of 54 samples were analyzed for diatom spores using light microscopy at 1000× magnification and immersion oil. Due to the difficulties in differentiating between the morphology of *Chaetoceros* resting spores, they were not separated to species' level. Concentrations of *Chaetoceros* resting spores were calculated as the numbers of valves per gram dry weight (gdw) (Battarbee and Kneen, 1982).

Single resting spore isolation from recent and several millennia-old resting spores

Only the resting spores which germinated were isolated. The single spores were isolated with care following the protocol of Throndsen (1978) where each sample was diluted with ~1 ml of sterile ddH$_2$O in a 30 mm × 15 mm petri dish and a drop of the solution was placed on a concave microscope slide. Individual resting spores were isolated through manual suction using 20–40 μL drawn-out disposable pipettes and examined on a concave microscopic slide with a CETI Versus (Medline UK, Chalgrove, UK) inverted microscope with a 10× objective and a wide focal 40x eyepiece with a total magnification of 400×. The isolated resting spore possibly with associated contaminants was transferred to a new double-distilled water droplet. This isolation and transfer were repeated 2 to 5 times to remove any contaminants. Individual resting spores were then isolated for the final time and transferred to a 30 mm × 15 mm petri dish containing culture media of Guillard's F/2

medium (20 ml) added to 1L artificial seawater (Tropic marine®, Wartenberg, Germany).

Germination and germination rate of the recent and several millennia-old resting spores

The resting spores were germinated in 30 mm × 15 mm petri dishes containing artificial seawater medium with salinities ranging from ~10.5 to 11 PSU at temperatures ranging between 15° and 18°C. A light intensity of 125 uE m^{-2} s^{-1} and a 12:12 light-dark regime was provided by fluorescent lamps in a Conviron growth chamber (E15, CMP3244, Conviron Europe, Ltd., Isleham, UK) (Delp et al., 2009).

The germination rate of the resting spores was estimated by counting the number of spores that germinated in a field of view of the microscope using the standard direct-count method (Kirchman et al., 1982; Pandey et al., 2013). The germination rate observed in 12 to 21 microscopic fields of view was recorded for each sample at a total magnification of 400×. The average germination rates for the recent and ancient resting spores were calculated by performing a one-way ANOVA and post hoc Holm-Bonferroni test (Holm, 1979) to compare the germination rates of the revived spores of recent age with the germinated rates of the MCA and HTM ages and also between the MCA and HTM ages. In the one-way ANOVA the age of the resting spores was the fixed effect and the germination rate the dependent variable using R (R Core Team, 2017). A post hoc Holm-Bonferroni test was then performed for pairwise comparisons among the three ages and to counteract the problem of multiple comparisons.

Growing the unialgal *Chaetoceros* cultures

Unialgal cultures of *Chaetoceros* from revived individual resting spores from the last century were established from an inoculum from the petri dish, which was transferred to 50 ml angled neck Nunc® EasY Flasks™ with vent caps (Thermo-Fisher Scientific, Waltham, Massachusetts, USA). The flasks were filled with the Guillard's (F/2) Marine Water Enrichment Solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) which contained silicate and grown in growth chambers with controlled temperature (15°–18°C) and a 12:12 light-dark regime. The light intensity was 125 uE m^{-2} s^{-1} and was provided by fluorescent lamps. The culture medium was prepared by adding 20 ml of the Guillard's F/2 medium in 1L of artificial seawater. The cultures were subcultured every two weeks to maintain the culture.

Identification of *Chaetoceros* species using light and scanning electron microscopes

The germinated *Chaetoceros* species was cleaned in hydrogen peroxide according to the method described in Battarbee (1986). Samples prepared for light microscope analysis were dried onto a coverslip and mounted in Naphrax™. Qualitative analysis was carried out at Södertörn University in Huddinge, Sweden with an Olympus BX51 light microscope using Nomarski differential interference contrast with a magnification of 1000× and oil immersion. To study the taxon in higher magnification we used a FEI Quanta 650 field-emission scanning electron microscope (FEI, Hillsboro, Oregon, USA) with a secondary electron detector hosted at the Department of Geosciences, Swedish Museum of Natural History, Stockholm, Sweden. Cleaned samples were dried onto a stub and sputter-coated in gold before being studied in magnifications between 6000 and 23,000×. Diatom species' identification followed Cleve-Euler (1951), Johansen and Boyer (1995), Johansen and Rushforth (1985), Kipp et al. (2021), Krammer and Lange-Bertalot (1991), Li et al. (2016), Reinke (1984), Rushforth and Johansen (1986), Snoeijs and Kasperovičienė (1996), and Wujek and Graebner (1980).

Identification of the *Chaetoceros* species with DNA sequences

DNA isolation from diatom cultures of recent resting spores

In this study precaution against contamination was taken in every step from sampling to PCR. For the DNA extraction and amplification, care was taken to avoid contamination and preparations were conducted in a PCR-free environment. Total DNA was extracted from each *Chaetoceros* culture (based on revived spores from the last century) as described below. A total volume of 25 ml of the cell cultures was centrifuged at 2500 rpm for 10 min in a 50 ml Falcon tube, 20 ml of the supernatant was discarded using a syringe. The remaining 5 ml was centrifuged again at 2500 rpm for 5 min, 4 ml of the supernatant was then discarded. The remaining 1 ml was spun for 2 minutes. The supernatant was discarded. Genomic DNA was extracted using a modified chloroform protocol with the addition of cetyltrimethylammonium bromide (CTAB), as described by Zuccarello and Lokhorst (2005). The culture pellet was ground with a microfuge pestle in 500 μL of CTAB extraction buffer (2 % CTAB, 0.1 Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 1% PVP), 2 μL RNAse A (100 mg/ml), and 5 μL Proteinase K (20 mg/ml) and the tubes were incubated at 55 to 60°C for 30 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed. The tubes were then spun at 12,000 g for 5 to 10 min. The supernatant was removed to a new tube, avoiding interface. This step was repeated. DNA was precipitated in 100% ice-cold isopropanol, each tube was inverted and placed at room temperature for 30 min. The tubes were spun for 20 min at 12,000 g. The DNA pellet was washed in 500 μL of 70% ethanol and the tubes were spun for 5 min. The supernatant was poured out and the DNA pellet was air-dried.

TABLE 1 AMS radiocarbon dates and calibrated ages of bulk sediment samples from Expedition 347, site M0063, holes C and D

Laboratory ID	Site and hole	Sample ID IODP	Depth (mcd)	^{14}C Age	Error	Calibrated age 1-sigma range		
						mean	min	max
Core top			0			−63		
Beta 418207	M0063C	347-25772	5.22	1760	30	817	333	1301
Beta 418208	M0063C	347-25773	5.72	2050	30	1063	569	1556
Beta 418209	M0063C	347-25774	6.22	1900	30	962	518	1405
Beta 418041	M0063D	347-28991	15.65	4590	30	4129	3457	4801
Beta 418042	M0063D	347-29274	17.29	5120	30	4802	4150	5453
Beta 418043	M0063D	347-29319	20.13	5480	30	5237	4585	5889
Beta 418046	M0063D	347-30771	27.45	7380	30	7316	6800	7833

The DNA pellet was suspended in 50 µL of 0.1 X (0.1 mM EDTA, 1 mM Tris) TE buffer. The quality of the DNA extraction was assessed by visualizing the products on a 1.5% agarose gel and the DNA concentration was evaluated with a nanodrop. The DNA was then frozen at −80°C for subsequent PCR and sequencing reactions.

PCR and sequencing

For the PCR reaction, 25 µL of reaction mixture contained as a final concentration, the two primers at 15 pM each; 5 to 20 ng DNA template; 0.2 mM each dATP, dCTP, dGTP and dTTP; 2.5 µL of 10× PCR buffer for AmpliTaq (25 mM $MgCl_2$ concentration); and 0.1 units of AmpliTaq DNA polymerase (ThermoFisher Scientific). The PCR condition was as follows: (1) an initial step at 94°C for 2 min followed by 30 cycles with a denaturation temperature of 94°C for 30 s; (2) an annealing temperature of 53°C for 30 s; and (3) an extension temperature of 72°C for 1.5 min. The PCR product was assessed by visualizing on 1.5% agarose gel. Four universal primer pairs from nuclear ribosomal RNA (small subunit, SSU) and two specific primers for chloroplast DNA (*rbcL*) from three unialgal cultures from recent spores were amplified using the primers published in Lee et al. (2013) (Appendix S1). Sanger sequencing was performed on the PCR products (Macrogen Europe, Amsterdam, Netherlands) of six nuclear ribosomal RNA (small (SSU) and chloroplast DNA (*rbcL*) markers which was used to amplify *Chaetoceros* species using the primers published by Lee et al. (2013) (Appendix S1). These markers were selected as these genes were tested and identified as taxonomic markers for centric diatoms in a previous study by Lee et al. (2013) (Appendix S1).

Analysis of DNA sequences

The similarity of the sequences was determined by using the basic local alignment search tool (BLAST) of the National

TABLE 2 Calibrated ages modelled for the diatom samples analyzed of MCA and HTM ages

Site and hole	Sample ID IODP	Depth (mcd)	Calibrated age 1-sigma range		
			median	min	max
M0063C	347-39293	5.42	810	516	1123
M0063C	347-39299	6.02	1104	816	1421
M0063C	347-39300	6.12	1154	865	1470
M0063C	347-39301	6.22	1203	912	1521
M0063C	347-39302	6.32	1254	958	1574
M0063D	347-29645	21.63	5687	5310	6062
M0063D	347-29748	21.83	5729	5352	6101
M0063D	347-29750	23.74	6113	5740	6489
M0063D	347-30184	24.07	6180	5805	6557
M0063D	347-30204	26.05	6582	6209	6959

Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA). The program BLASTN version 2.9.0+ (Zhang et al., 2000; Morgulis et al., 2008) and the nucleotide collection (nr/nt) database was used. Sequences were aligned by using Molecular Evolutionary Genetics Analysis version 6 (MEGA6) as was the single nucleotide polymorphism (SNP) analysis (Tamura et al., 2013).

RESULTS

Dating and age modelling

Three sediment samples of MCA age and four samples of HTM age were radiocarbon dated and used for age modelling to assign an age to all individual samples used for the resurrection study (Tables 1 and 2). Radiocarbon dating together with stratigraphic time markers for the youngest sediment

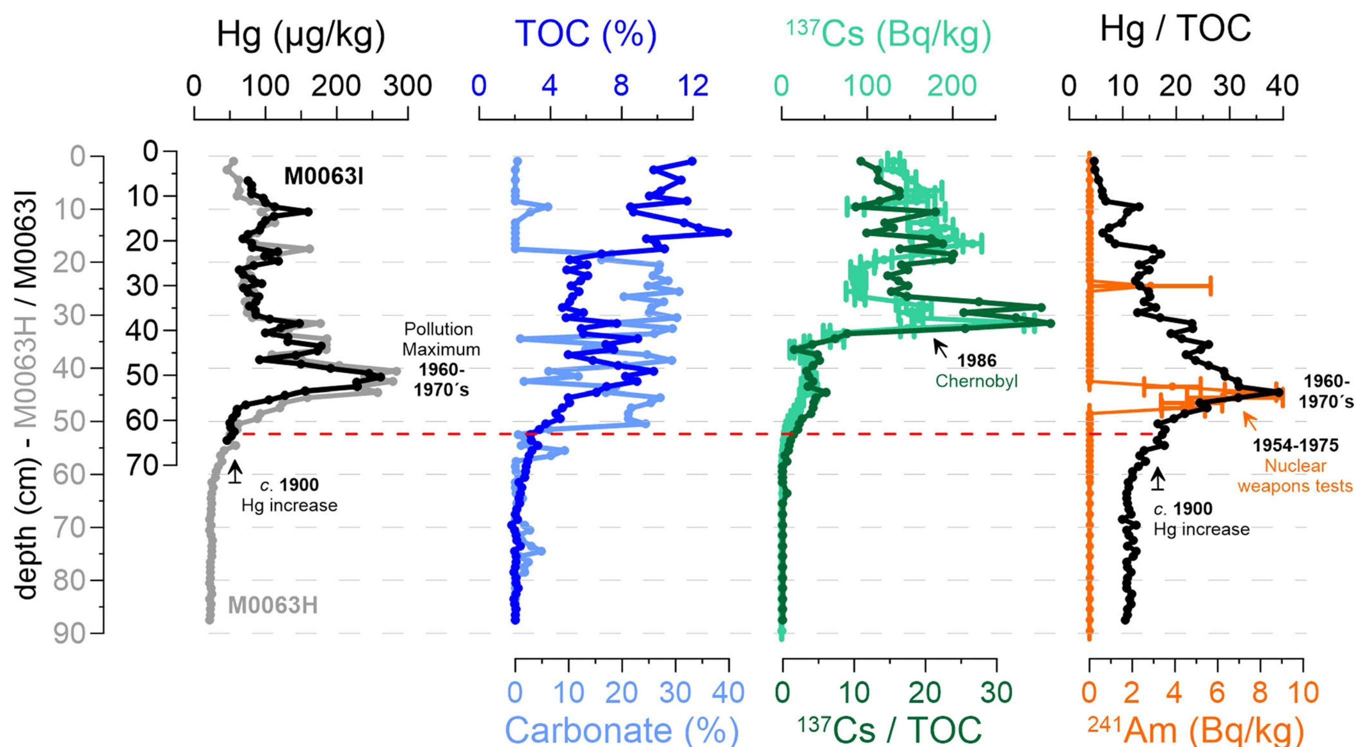

FIGURE 2 Stratigraphic time markers in cores M0063I (mercury - Hg) and M0063H (Hg and artificial radionuclides ^{137}Cs and ^{241}Am) following the approach described in Moros et al. (2017). Core correlation is based on Hg downcore profiles in M0063I (black) and M0063H (grey) which are shown versus respective core depths. In addition, total organic carbon (TOC, blue) and carbonate (light blue) data of M0063H are shown. Hg (dark green) and ^{137}Cs (black, right) are normalized to TOC as the manganese-carbonate layers dilute the signals. Horizontal dashed red line marks an age of ca. AD 1940 which can be assigned to this depth

(Figure 2) showed that our samples were from three distinct time intervals from present time to ~6600 cal. yr BP. The sediment from the short gravity core M0063I with an anticipated age of the last century could be assigned an age of ca. 1940 to 1935 (i.e., ~0 to 80 years) at 63 cm, based on the stratigraphic time markers identified (Figure 2). Modelled ages of the sediment samples used for the resurrection study are: ~1250 to 800 cal. yr BP from MCA age (M0063C 5.42 to 6.32 mcd); and ~6600 to 5700 cal. yr BP of HTM age (M0063D 21.63 to 26.05 mcd) (Table 2). Age modelling made it possible to estimate the calendar years age for all levels analyzed except for the sample at 6.32 mcd which was outside the dated interval. A linear extrapolation from the sample at 6.22 mcd was used to estimate the age of this sample (Table 2). Age-depth models for analyzed sequences in the cores M0063C and M0063D are presented in Appendix S2.

Resting spore concentration across evolutionary timescales

The highest *Chaetoceros* spp. resting spore concentrations were analyzed in the sediments deposited during the HTM (~203 million resting spores per gram dry weight of sediment at 26 mcd), after which concentrations decreased gradually with distinct peaks during MCA and present time (Figure 3). Preservation of diatom valves was very poor between ~21 and 8.5 mcd, however the heavily silicified resting spores were still found in some of the sediment samples in this location in relatively low concentrations. Due to possible dissolution, concentrations might not correspond to reconstructed production in this section. During the MCA age, resting spore concentrations increased to ca. 185 million at ca. 5.5 mcd, following which concentrations decreased again. The most recently deposited sediments revealed a pronounced increase in *Chaetoceros* spp. resting spore concentration (~146 million/gdw). The high spore concentration in the recently deposited sediments is only comparable to the concentrations found during the HTM and MCA in our record.

Germination of recent and millennia-old resting spores

We revived resting spores from the three time periods: 0 to 80 years (last century); 1250 to 800 cal. yr BP (MCA); and ~6600 to 5700 cal. yr BP (HTM). On an average the time taken for the resting spores to germinate was observed to be consistent for all three ages and ranged from three hours to 2 to 3 days at temperatures ranging from 15° to 18°C and at salinity of ~10.5 to 11 (Figure 4). So, the time taken to germinate did not change with increasing age of the spores.

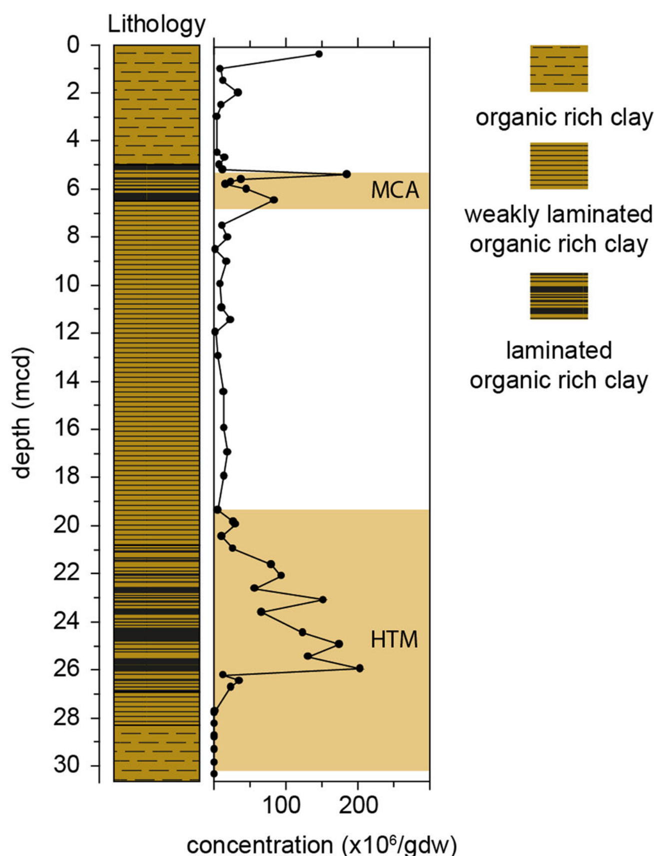

FIGURE 3 Lithology and *Chaetoceros* spp. resting spore concentrations (million valves per gram dry sediment) in the upper 30-meter sediments of IODP Expedition 347 hole M0063D (Landsort Deep). The high concentrations of MCA and HTM ages are recorded in laminated organic rich clays (equals to hypoxic bottom water conditions)

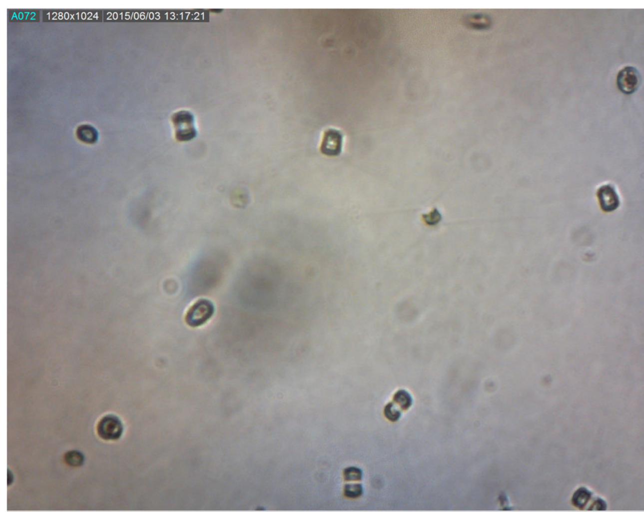

FIGURE 4 Germination of resting spores of *Chaetoceros muelleri* var. *subsalsum*

TABLE 3 Germination rate (%) of the resting spores from the three ages

Resting spores	Recent	Ancient (MCA)	Ancient (HTM)
n	22	12	5
Mean	41%	31%	12%
SE	8.7	8.9	6.7
Significance (One-way ANOVA)	$P = 0.0006$		
F (One-way ANOVA)	9.22		
Post hoc Holm-Bonferroni tests:			
Recent vs. MCA	$P = 0.052$		
Recent vs. HTM	$P = 0.0008$*		
MCA vs. HTM	$P = 0.0097$*		

Note: *Indicates that the *P*-value of the germination rate between the spores of the recent and HTM ages and the MCA and HTM ages were significant when subjected to the Holm-Bonferroni correction.

Germination rate and viability of recent and millennia-old resting spores

The number of resting spores which germinated from the three time periods varied. A greater number of the younger (~0 to 80 years and 1250 to 800 cal. yr BP) resting spores germinated as compared to the oldest (~6600 to 5700 cal. yr BP) resting spores. On an average, the percent germination rate of the recent resting spores was the highest at 41 ± 8.7 (mean ± SE, n = 22), while the germination rates of the resting spores of the MCA and HTM ages were 31 ± 8.9 (mean ± SE, n = 12) and 12 ± 6.7 (mean ± SE, n = 5), respectively (Table 3). A one-way ANOVA showed significant differences in the germination rate of the recent and ancient spores of the MCA and HTM ages ($P = 0.0006$, F = 9.22) when comparing the germination rates of all three ages (Table 3). Post hoc test using Holm-Bonferroni corrections showed significant differences ($P = 0.0008$) when pairwise comparisons were made between the germination rates of the resting spores from recent and the HTM ages; and between the resting spores of the MCA and HTM ages ($P = 0.0097$). However, no significant difference in the germination rate was found between the recent spores and the MCA age ($P = 0.052$) when subjected to Holm-Bonferroni correction.

Germination and reproduction of the recent and millennia-old resting spores

The resting spores isolated from the last century (0–80 years) reproduced and could be grown in culture but the old spores (~1250 to 800 and ~6600 to 5700 cal. yr BP) of the

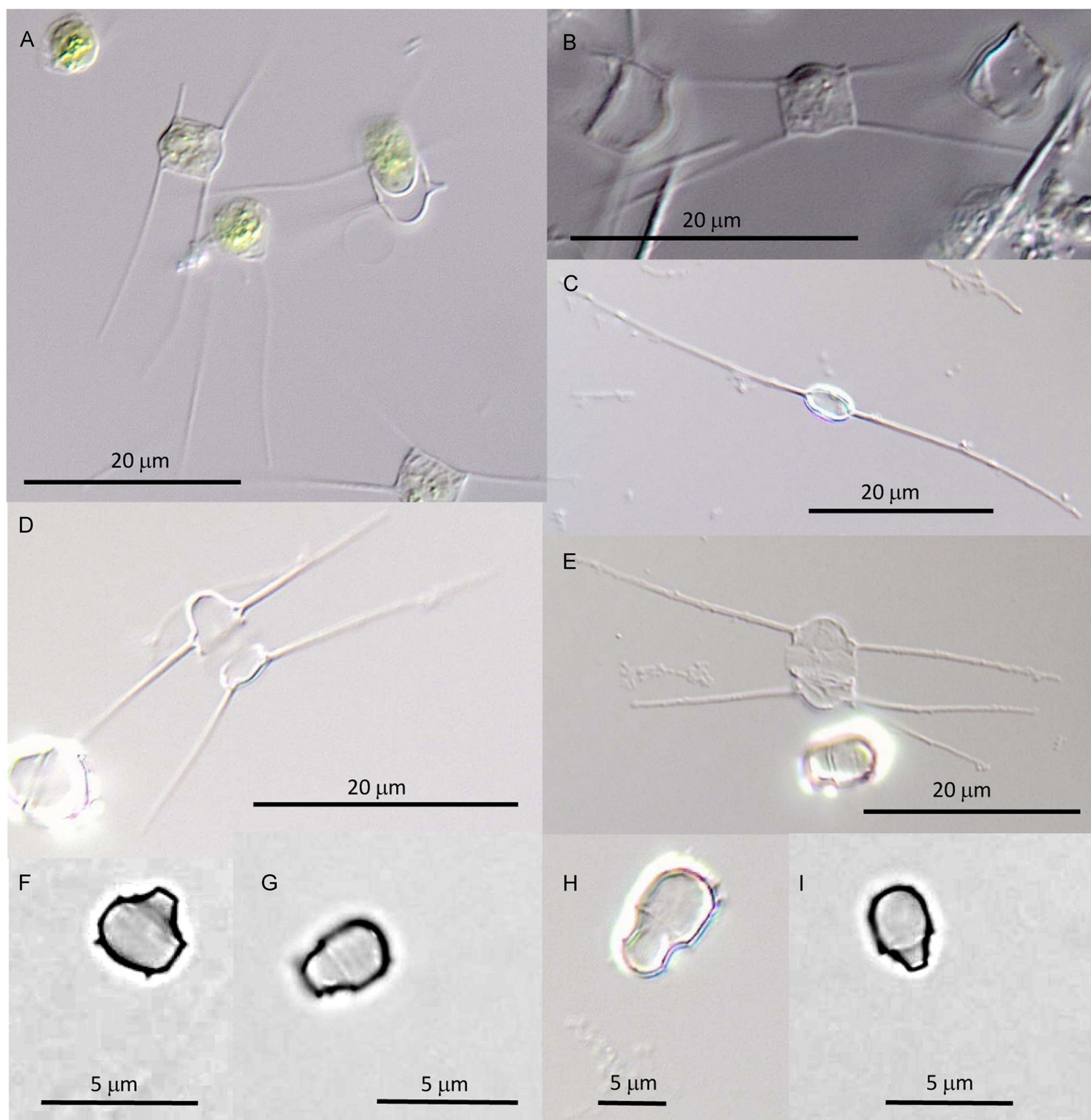

FIGURE 5 Light micrographs of *Chaetoceros muelleri* var. *subsalsum* revived from the sediment at 10 to 11 cm depth. All micrographs show cleaned samples except for A and B. (A–B) whole cells in girdle view from the culture. (C) Smooth solitary vegetative cell in valve view. (D–E) Vegetative cells in girdle view with convex valve shape. Note that both vegetative cells and resting spores are visible. (F–I) Resting spores in girdle view showing various characteristic morphologies. In F two spines are visible on the valve face

MCA and HTM age germinated but could not be grown in cultures. The time taken for the recent and ancient spores was the same ranging from three hours to 2 to 3 days, but the germination rate (%) of the ancient spores decreased with increasing age (see section on germination rate) (Table 3).

Identification of the *Chaetoceros* species based on microscopy and DNA sequences

Based on the study of cleaned material using light and scanning electron microscopy (Figures 5 and 6), we identified the germinated *Chaetoceros* resting spores that we

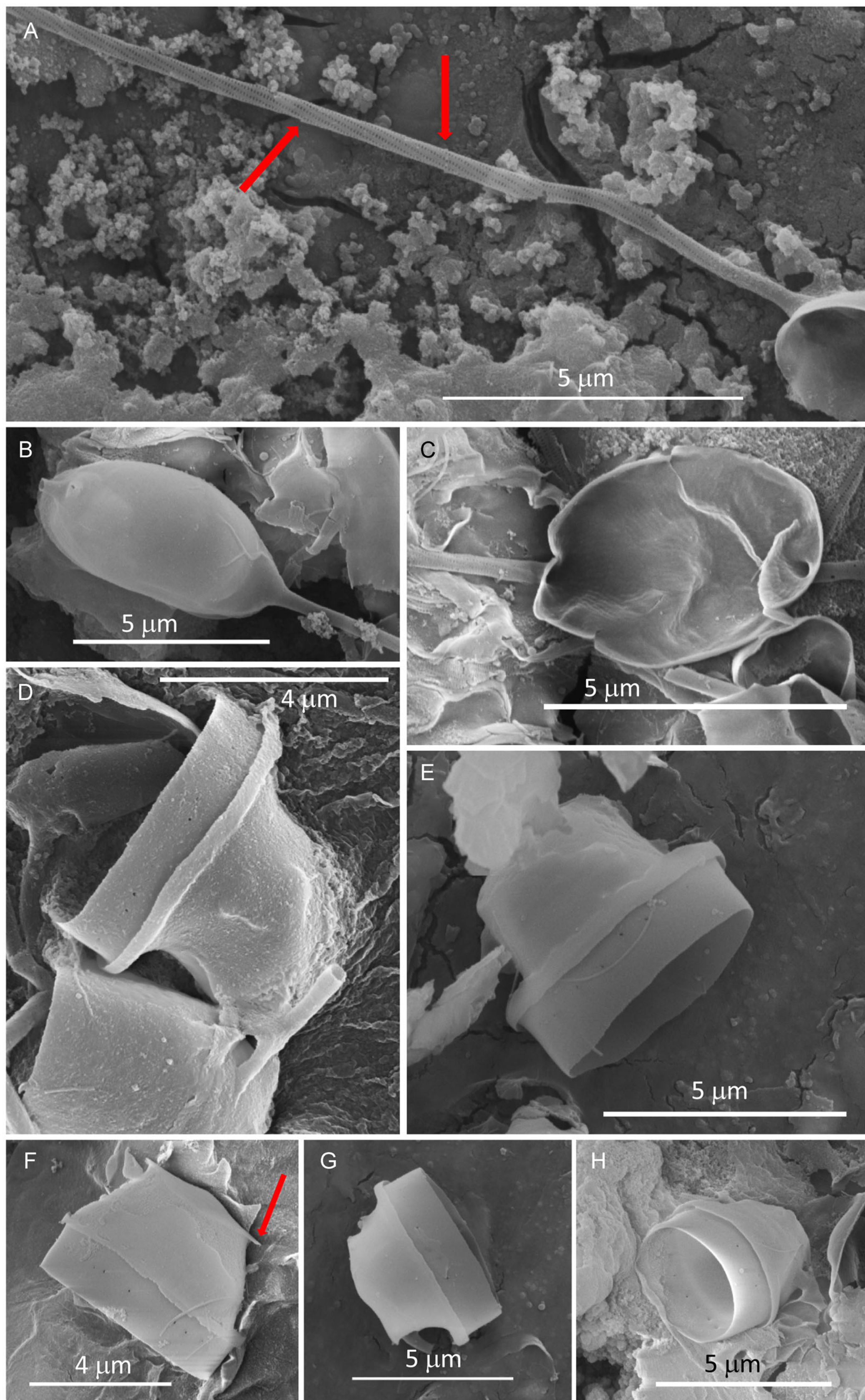

FIGURE 6 Scanning electron micrographs of *Chaetoceros muelleri* var. *subsalsum* revived from sediments at 10 to 11 cm depth. (A) Small spines (red arrow) and fine puncta in a spiral pattern visible on the seta. (B) Vegetative smooth valve lacking processes. (C) Valve ornamentation composed of an anastomosing pattern and display of hollow setae. (D–H) Exterior view of resting spores with various shapes and F showing a valve with spines (red arrow)

were able to culture as *Chaetoceros muelleri* var. *subsalsum* (Lemmerman) J.R. Johansen and Rushforth (originally described as *C. muelleri* var. *subsalsum* but referred to as *Chaetoceros muelleri* var. *subsalsus* in AlgaeBase, website www.algaebase.org). The vegetative cells are smooth, without ornamentation, and elliptical in valve view (Figures 5C and 6B) with long setae (Figures 5A–E and 6A) and contain one chloroplast (Figure 5A). The germinated vegetative cells have a valve length of 4 to 6 μm (mean 4.9 μm) excluding setae and the resting spores valve length range between 3 and 8 μm (mean 4.9 μm). Setae are slightly curved and oriented in similar directions on opposite valves in valve view (Figure 5C; cf. Johansen and Boyer, 1995, figure 1; Li et al., 2016, figure 18). The setae are hollow (Figure 6C), have fine puncta in a spiral pattern and rows of small spines (see red arrows in Figure 6A) which fit the descriptions of *C. muelleri* in Reinke (1984) and *C. muelleri* var. *subsalsum* in Johansen and Rushforth (1985). Our germinated specimen is showing an anastomosing ornamentation pattern in valve view (Figure 6C), as reported to occur in *C. muelleri* var. *subsalsum* (Johansen and Rushforth, 1985, their figure 36). Another feature seen in some of our specimens are two spines on the exterior of the resting spores (Figures 5F and 6F) also described in Johansen and Rushforth (1985). Furthermore, analysis of chloroplast *rbcL* gene and nuclear rRNA molecule (SSU) sequences obtained from unialgal cultures also helped identify the species in this study as *Chaetoceros muelleri* based on DNA sequences.

Extraction and amplification of DNA from unialgal cultures of recent resting spores

High quality DNA with concentrations ranging from ~100 to 235 ng/ul with a A260/A280 ratio of ~1.7 to 2 from cultures of the germinated resting spores of the last century (~0–80 years) were obtained. Six primer pair combinations of nuclear rRNA (SSU) molecules and chloroplast genes (*rbcL*) from three unialgal cultures from recent spores used for DNA amplification yielded up to 980 bp long sequences (Appendix S3).

DNA analysis of sequences from unialgal cultures of recent resting spores

Sequences for chloroplast genes, *rbcL* (up to 980 bp) and nuclear rRNA molecules, SSU (up to 550 bp) were generated from three sediment revived recent resting spore populations of *Chaetoceros* (Appendix S3). BLASTN searches of *rbcL* gene sequences generated in this study against complete chloroplast genome of *Chaetoceros muelleri* resulted in up to 99% query cover and up to 97% sequence similarity (Appendix S3). BLASTN searches of SSU markers sequences from this study against partial sequences of SSU markers in the database resulted in up to 100% query cover and up to 99% sequence similarity (Appendix S3). Sequence alignment and BLAST search of the nuclear ribosomal RNA (small subunit [SSU]) and chloroplast *rbcL* confirmed that the sequences were from the *Chaetoceros muelleri* (Appendix S3) which was consistent with the morphological identifications of our specimens. SNP analysis identified 4 to 15 SNPs in the three unialgal cultures (Appendix S4).

DISCUSSION

This study demonstrated that: (1) high concentrations of resting spores were observed in three ages (recent, MCA, and HTM); (2) radiocarbon dating along with stratigraphic time markers determined the ages of the sediments; (3) successful germination and the germination rates of recent (~0–80 years) and ancient resting spores (~6600–800 cal. yr BP) (Figure 4, Table 3); (4) identification of the taxon (*Chaetoceros muelleri* var. *subsalsum*) from the morphology of viable germinated resting spores (Figures 5 and 6) using light and scanning electron microscopy; (5) identification of the species from the DNA sequenced from unialgal cultures of the recent spores (~0–80 years) (Table 3); (6) SNP analysis of the populations revived from the recent spores suggested genetic variation among the populations (Appendix S2); and finally (7) the Baltic Sea as an excellent historical environmental record where resting spores are preserved and possibly revived to study long-term effects of environment on species' adaptation.

Our study has shown that the resting spore concentrations of the marine diatom *Chaetoceros* are very high during three ages (last century, MCA, and HTM) which correspond to times with high primary production, high sea surface temperatures (HTM, MCA), eutrophication (last century), and result in hypoxia (Zillén et al., 2008; Funkey et al., 2014). Recent studies have shown that increased salinity in the Baltic Proper caused increased hypoxic conditions and higher productivity of diatoms during the warm HTM conditions (van Wirdum et al., 2019) that also prevailed during MCA (E. Andrén et al., 2020). Warm climate creates a stronger stratification in the water mass resulting in hypoxic bottoms, but also enhances phosphorus recycling and sustaining cyanobacterial blooms which contribute to increase overall available nutrient concentrations (Funkey et al., 2014; Karlson et al., 2015). Hence, revived diatom resting spores from the Baltic Sea can be used to address questions about the impact of human-induced perturbations and climate change on life on Earth.

To the best of our knowledge this is the first study reviving thousands-of-years old (~6600–800 cal. yr BP) diatoms from sediments of known age. Previous studies on the diatom *Skeletonema marinoi* Sarno and Zingone showed that the sediments were dated to be a century old (Härnström et al., 2011). Other studies on the persistence of diatoms in marine sediments have reported germination of viable resting stages from sediment layers ~30 to 40 cm below the sediment surface which were estimated to be 175 to 275 years old (based on the sedimentation rates of

1.2–1.5 mm per year) (Stockner and Lund, 1970). Therefore, the ability to revive recent and ancient resting spores of *Chaetoceros* of unprecented age and identify *Chaetoceros* taxa from the morphology of vegetative cells will improve the scope of paleoecological studies (Witak et al., 2011).

Interestingly, our results indicate a different germination rate compared to that of the diatom species *Skeletonema marinoi*. The germination of resting spores of *Skeletonema marinoi* from sediment layers older than a few decades can take weeks to months (Härnström et al., 2011) while both the recent and ancient *Chaetoceros muelleri* var. *subsalsum* spores in this study germinated in three hours to 2 to 3 days, which is a surprising and promising result. However, it was observed that although the time taken for the recent and ancient resting spores to germinate remains the same (three hours to 2 to 3 days) the germination rate (% of germination) of the ancient spores decreases with increasing age (Table 3).

We also found that the older spores (~1250–800 cal. yr BP) could be germinated but could not be grown in cultures. It is possible that the older spores had the ability to survive but were unable to reproduce. The lower germination rate of the ancient spores indicates a trade-off between the longevity and viability of the spores and reproductive ability of the spores. A one-way ANOVA showed that the germination rates varied significantly ($P = 0.0006$) when all three ages (Table 3) were compared, which also suggests that just to remain viable over thousands of years has been a challenge for the species. Post hoc Holm-Bonferroni tests showed significant differences in the germination rates between the recent and MCA ages ($P = 0.03$); recent and HTM ages ($P = 0.01$), but not between the MCA and HTM ages (Table 3).

Previous attempts to use resting spores for paleoenvironmental reconstructions by assigning them to species met with limited success as most of the resting spores were difficult to classify (Ishii et al., 2011; Witak et al., 2011), severely hindering our understanding of paleoproductivity and the effect of environmental change on species. Resting cells of diatoms are virtually identical to vegetative frustules in thickness as well as shape and pattern (McQuoid and Hobson, 1996). Previous studies on the diatom *Skeletonema* have shown that the morphology of the resting cells and germinated vegetative cells is indistinguishable (Itakura et al., 1992), whereas the resting spores and germinated vegetative cells of *Chaetoceros* species can be distinguished based on morphology.

Chaetoceros muelleri unlike most *Chaetoceros* taxa is a solitary-living species with its vegetative cells characterized by a convex or flat valve surface, and its resting spores smooth with one valve bowed and the other protruded and truncated (Johansen and Rushforth, 1985). The above characteristics fit with the vegetative cells (Figure 5A–E) and resting spores (Figure 5F–I) found in our culture. There are few solitary *Chaetoceros* species which presently occur in the Baltic Sea (Hällfors, 2004; see also website http://nordicmicroalgae.org/) namely *C. danicus* P.T. Cleve, *C. ceratosporus* Ostenfeld, *C. gracilis* Schütt, *C. muelleri* Lemmermann, *C. simplex* Ostenfeld, *C. tenuissimus* Meunier, *C. throndsenii* (Marino et al.) Marino et al., *C. vistulae* Apstein, *C. wighamii* Brightwell/*C. fallax* Proschkina-Lavrenko, but only three taxa without or unclear status of processes on valves: *C. muelleri* var. *subsalsum*, *C. minimus* (Levander) Marino et al., and *C. calcitrans* (Paulsen) Takano. Johansen and Boyer (1995) exemplify the complexity in using morphological characteristics to separate *Chaetoceros* resting spores to species' level and show that variability in shape of the spores is influenced by the stage in life cycle when the spore is formed. In Johansen and Boyer (1995) the spores of *C. muelleri* var. *subsalsum* lacked spines, but the authors do not exclude the possibility that spines may occur in field material. Furthermore, the position of the setae in a complete vegetative cell is an important morphological characteristic to separate *Chaetoceros muelleri* var. *subsalsum* (oriented in similar direction in valve view and in same focal plane in girdle view, Figure 5C–E) from *C. transisetus* Johansen & Boyer and *C. coloradensis* Li, Kociolek & Gao (Johansen and Boyer, 1995; Li et al., 2016). By using the dichotomous key on specific morphological characteristics in solitary *Chaetoceros* taxa without processes presented by Li et al. (2016) when they described the new inland species *C. coloradensis*, our species can be identified as *Chaetoceros muelleri* var. *subsalsum* (Lemmermann) J.R. Johansen & Rushforth. Reinke (1984) describes *C. muelleri* as weakly silicified and delicate with partially collapsed frustules, which is also the case for our taxon (Figure 6A–C), making determination and documentation of species more difficult. To separate the variety *subsalsum* from the nominate variety *Chaetoceros muelleri* var. *muelleri* we need to see the lack of processes in spores of *subsalsum*, but also the smaller cell size of both the vegetative cells and resting spores (Johansen and Rushforth, 1985; Johansen and Boyer, 1995). The nominate variety has processes on two, one, or neither valve which makes the separation between the varieties difficult. According to Rushforth and Johansen (1986) the two varieties of *C. muelleri* can only be separated with confidence when a large population of cells is examined. We found no processes in the population we examined, and the cell sizes are within the smaller range. The variety *subsalsum* is furthermore less frequently found living colonially (Johansen and Rushforth, 1985), and our taxon was all solitary-living. Based on this we concluded that we have the variety *subsalsum*, but the number of specimens examined (n = 25) might not be numerous enough to rule out the nominate variety. *Chaetoceros muelleri* var. *subsalsum* has been reported as non-indigenous species in the Great Lakes in North America where it was probably introduced via ballast water (Johansen and Rushforth, 1985; Mills et al., 1993; Kipp et al., 2021). In the Great Lakes *C. muelleri* var. *subsalsum* is recorded where there is a high nutrient level in the water, but salinity is the primary factor controlling its growth rate (Kipp et al., 2021). In the checklist of Baltic Sea Phytoplanktic species; *C. muelleri* (including var. *subsalsum*) is recorded in nearly all subareas and considered a warm-water species found in low salinity and mainly eutrophic waters

(Hällfors, 2004). According to the intercalibration guides of Baltic Sea diatoms *Chaetoceros muelleri* var. *subsalsum* is presently rarely recorded in coastal plankton in the Askö area (the coast closest to our sampling site) in a salinity of 6 to 7 PSU but is not recorded in the open Baltic Proper (Snoeijs and Kasperovičienė, 1996). The lack of the variety *subsalsum* in the open Baltic Proper is most likely an artifact of that it has been reported as *C. muelleri* (Hällfors, 2004). Hence, by reviving resting spores deposited in sediments and examining their morphology under LM and SEM we have identified the species as *Chaetoceros muelleri* var. *subsalsum*. The *rbcL* and SSU sequences obtained in our study also confirmed that the species is *Chaetoceros muelleri.* Previous studies have also successfully identified diatoms based on *rbcL* genes (Li et al., 2016; Vasselon, Domaizon et al., 2017; Vasselon, Rimet et al., 2017). The classification of the species to the variety level has been possible based on morphology. However, the classification to the species level with *rbcL* and SSU sequences can be attributed to the incompleteness of the reference databases (Vasselon et al., 2018). In addition, the resting spores in our study germinated when we tested at temperatures as high as 31°C which also suggested that the species was *Chaetoceros muelleri* as has been reported in other studies (Blinn, 1984; McGinnis et al., 1997). Previous studies on several other *Chaetoceros* species showed that the other *Chaetoceros* species studied did not germinate at temperatures >25.3°C (Hollibaugh et al., 1981). Thus, the identification of the revived resting spores down to the species' and varietal level as *C. muelleri* var. *subsalsum*. for the first time provides evidence of revival of resting spores which are several millennia old.

We observed good preservation of the resting spores in hypoxic sediments from the Landsort Deep. Anoxic conditions help in preserving the spores (Ellegaard and Ribeiro, 2017) possibly reducing their metabolic activity through time. Also, it is possible that the changes in the genome due to climatic or environmental changes over a thousand years could have affected the ability of the ancient spores to reproduce as we could not establish cultures from the ancient spores. It has also been observed that microalgal species thrive in presence of bacteria in cultures (Fitzsimons and Smith, 1984; Mouget et al., 1995). Bacteria produce vitamins and organic growth factors (Ukeles and Bishop, 1975) and can change the pH of the growth medium (Caldwell, 1977). Algae can also release large quantities of organic compounds (Hellebust, 1974), which are used by bacteria (Bell, 1983), while the bacteria can be a source of CO_2 during algal growth primarily during periods of carbon limitation (Marshall, 1989). The algal-bacterial relationship especially during algal blooms and eutrophication is also dependent on the abundance of bacteria around the algal cells which will determine the dissolved oxygen levels due to that produced photosynthetically by algae or consumed by bacteria. Furthermore, the release of stimulatory or antagonistic compounds by bacteria could increase or inhibit algal growth (Mouget et al., 1995). Hence, in addition to changes in the genomic signature presence of certain phycosphere-associated bacteria could be facilitating the establishment of cultures from revived spores from recent sediments while the absence of certain bacteria could be hindering the establishment of cultures from the revived ancient spores.

Previous studies especially in humans has revealed that contamination is a continuous problem using ancient DNA (Hofreiter et al., 2001). Hence, the availability of high-quality DNA from sediment-revived spores in a system so well-preserved where the chances of contamination are minimal as opposed to systems involving humans is like a dream come true and provides immense potential for future microevolutionary studies. The successful DNA extraction, amplification, and sequencing of the plastid-encoded *rbcL* and nuclear-encoded SSU rRNA genes from unialgal cultures of the recent spores (~0 to 80 years) reveals that the DNA in the sediment-revived resting spores are well-preserved suggesting the possibility for further studies using modern DNA sequencing methods to examine changes in genomic signatures in *Chaetoceros* species due to natural climate change and eutrophication (Orsini et al., 2013). Furthermore, the identification of 4 to 15 SNPs (Appendix S2) in the three unialgal cultures from recent spores reveals the genetic variation between *Chaetoceros* strains isolated from different strata in the cores.

Previous studies have shown that Baltic Sea populations of several species (e.g., *Zostera marina* L., *Fucus vesiculosus* L., *Cladophora rupestris* (L.) Kützing) displayed significantly reduced genetic diversity compared to the Atlantic and North Sea populations, following the salinity gradient (Johannesson and André, 2006). A study on the diatom *Skeletonema marinoi* showed significant differences in the growth of low and high native salinity isolates indicating local salinity adaptation (Sjöqvist et al., 2015). Also, local adaptation to changing salinity conditions was also observed in the barnacle species *Balanus improvisus* Darwin in the Baltic Sea (Wrange et al., 2014).

However, earlier studies predicting how evolution will shape the genetic architecture of populations coping with present and future environmental challenges have primarily relied on investigations through space, in lieu of time. Here, we show that it will be possible to examine the genetic diversity and mechanisms of evolutionary changes in *Chaetoceros* species across evolutionary timescales from the time the Baltic Basin became brackish about 8500 years ago. Adequate comprehensive studies in phytoplanktic species along

environmental gradients like salinity or temperature combined with experimental data are lacking and the results in this study shows that it is possible to do this over long timescales.

CONCLUSIONS

In this study we identify and present a unique model system, *Chaetoceros muelleri* var. *subsalsum* resting spores from the Baltic Sea. *Chaetoceros* resting spores are found

well-preserved throughout the brackish sediment stratigraphy in the open Baltic Sea due to a high sedimentation rate, high primary production, and hypoxic conditions. Hence, revived *Chaetoceros* resting spores can be used to increase the understanding of the evolutionary fate of the species on a temporal scale instead of just a spatial scale by studying genomic signatures in changing environmental conditions like salinity, stratification, and increased nutrient discharge caused due to climate change and human actions.

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AUTHOR CONTRIBUTIONS

The conceptualization of the ideas was done by E.A. and M.L. Formal analysis of the data was done by T.A., M.M., F.V.W., and A.S. Funding was obtained by E.A., M.L., and T.A. Investigation, as well as visualization and presentation was done by E.A., T.A., M.M., F.V.W., and A.S. Methodology was done by E.A., M.L., J.L., and A.S. Supervision, project administration, and resources were done by E.A. and T.A. Validation was done by A.S., E.A., T.A., and M.M. Writing of the original draft was done by E.A and A.S. Writing, review, and editing were done by E.A., A.S., J.L., M.M., F.V.W., and T.A.

DATA AVAILABILITY STATEMENT

The FASTA files of the sequence data are available on the National Center for Biotechnology Information (NCBI) databases (https://www.ncbi.nlm.nih.gov/genbank/) under accession nos. OK169542, OK169543, OK169544, OK169545, and OK169546. The raw germination data is available on FigShare at https://figshare.com/articles/dataset/Germination_data/16676719.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Appendix S1. Primers used for PCR and sequencing of rDNA and chloroplast genes in *Chaetoceros muelleri*.

Appendix S2. BACON generated age-depth models for the cores M0063C (top) and M0063D (bottom). Upper small panels are from left to right depict the MCMC (Markov Chain Monte Carlo) iterations, the distribution for the accumulation rate and the memory (green curve prior and grey histograms posterior). The lower panels show the calibrated ^{14}C dates (transparent blue) and the age-depth model. Based on the mean age for each depth, the red stippled line shows the best model and the darker grey indicates more likely calendar ages. The grey stippled line shows the 68% (1-sigma) confidence intervals.

Appendix S3. BLASTN results of the SSU and *rbcL* sequences.

Appendix S4. Frequency distribution of single nucleotide polymorphisms in the three unialgal populations.

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