Holocene DNA sequences – a dream come true

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Abstract

Microscopic marine planktons have played a major role in the Earth's ecosystem and accounts for half the globe's primary production. Predicting the fate of marine planktons is imperative to understanding how ocean life will change in future centuries and how organisms have adapted to changes in the past over long timescales (over thousands of years). Studies of revived resting spores of marine diatoms will serve as excellent proxies of environmental change in marine environments. Thus far, only about a century old marine diatom resting spores could be revived. This severely limited the use of diatom resting spores to investigate the effects of environmental perturbations over longer time scales. Here, we, for the first time report revival of thousands of years old resting spores from the deposits of ancient diatoms (*Chaetoceros*) in sub-seafloor sediments using a revival protocol that involved recreating the ancient environmental conditions (salinity, temperature and light). Our revived diatom resting spores samples range from recent (0-80 years) to ~7200 years old. Importantly, we also extracted the DNA from the ancient resting spores of *Chaetoceros* species from the Baltic Sea. Our findings will enable us to compare DNA sequence data obtained from these natural archives of resurrected organisms and provide predictive models to forecast evolutionary responses of natural populations to environmental changes resulting from natural and anthropogenic stressors, including climate change

Significance Statement

Our results address the important topic of adaptive evolution in marine species due to climate and environmental change induced due to anthropogenic perturbations. We present a new model system *Chaetoceros muelleri* which will help us address important evolutionary and long-term adaptation questions across evolutionary timescales. Our study reports (1) the revival of recent (0-80 years) to ancient (7200 years old) resting spores of *Chaetoceros* (2) the extraction of DNA and amplification of chloroplast and ribosomal genes from recent and ancient (~1300 years old) resting spores of *Chaetoceros* (3) Radiocarbon dating to determine age of sediments (4) Identification of species by reviving the resting spores (5) Baltic sea an excellent ecosystem to study long-term effects of environment on species adaptation

Introduction

Human domination of the Earth's ecosystems has accelerated in recent decades, to the extent that this period has been termed as the anthropocene (1). We have very little knowledge about how the Earth's biota will be affected by human-induced climate change (2), and understanding how

global biodiversity will respond to anthropogenic perturbations (2) has emerged as a major challenge to humanity. Human-induced environmental pollutants and nutrients that reach the aquatic environment through sewage effluents, agricultural and industrial processes are constantly contributing to environmental changes that drive adaptive responses and evolutionary changes in many taxa (3, 4). Oceans are at a greater risk due to both climate change and the dumping of industrial and human waste. Changes in species abundance with increased numbers of planktic, resistant, toxic, and introduced species due to nutrient enrichment and resulting symptoms of eutrophication, hypoxia, metal pollution and acidification (5) has resulted in significant loss of biodiversity in the marine environment. It remains a major challenge to predict how climate change will affect distribution, diversity and adaptive responses of different species (6). Indeed, our current inability to do so hampers our understanding of how the future ocean will function. In spite of recent advances in characterizing these organisms through global comprehensive surveys and advanced DNA sequencing technologies, our knowledge about their capacity to adapt to a changing environment is limited. A key to understanding the adaptive capacities of species over evolutionary time lies in examining the recent and millenia old resting spores buried in the sediments. So far, studies have only reliably examined a century old marine diatom resting spores (7).

Resurrection ecologists have long recognized sediments as sources of viable propagules ("seed or egg or resting spore banks") (8) with which to explore questions of community ecology, ecological response, and evolutionary ecology. Studies predicting how evolution will shape the genetic architecture of populations coping with present and future) environmental challenges has primarily relied on investigations through space, in lieu of time. Yet, dormant propagules in sediments are natural archives of population-histories from which adaptive trajectories along extended time periods can be traced. DNA sequence data obtained from these natural archives of resurrected organisms, combined with the currently available methods for analyzing both ecological and population genomic data can provide predictive models to forecast evolutionary responses of natural populations to environmental changes resulting from natural and anthropogenic stressors, including climate change (9). Resurrected organisms provide exceptional opportunities to study evolutionary processes and can be used to test paleo-proxies of environmental change (9, 10).

To systematically study the effects of anthropogenic perturbations over long time scales we need (1) a model organism whose resting spores have accumulated over a long period of time and (2) an ecosystem which has the deposits of the resting spores of the model organism and has been subjected to anthropogenic perturbations over a long period of time. Here we propose that the *Chaetoceros* resting spores in the Baltic sea is a good model system to study the impact of anthropogenic perturbations over long time scale, provided the resting spores from different time periods can be revived and their DNA can be extracted. Here we for the first time show that several millenia old resting spores can be successfully revived and their DNA extracted.

Baltic Sea ecosystem can store resting spores over millenia

We also hypothesized that the Baltic Sea ecosystem with its horizontal (salinity and temperature) and vertical environmental gradients (salinity, temperature and oxygen) (11) and a high sedimentation rate (which would result in quick burial of the spores) and abundance of multiple species of *Chaetoceros*, would be an ideal ecosystem for sampling sediments for well preserved spores for our sediment revived resting spore studies. The water consists of a mixture of inflowing marine water from North Sea and the Danish straits and freshwater runoff from a large drainage area four times the size of the water surface (12). This results in a long salinity gradient ranging from the transition zone in Kattegatt (12-30) and the Danish straits (10-23), to the Baltic Proper (5-7.5) and the almost freshwater Bothnian Bay (2-4) (13) (Fig. 2). 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 (14). The geological history of the Baltic Basin makes the Baltic Sea very young from an evolutionary perspective, and only a few species have adapted to its brackish environment (8). The Littorina Sea (7500-3000 cal yr before present (BP)) reached a maximum surface water salinity of 12-13 in the Baltic Proper, compared to

present day 6-8 (15,16). The higher salinity coincides with the Holocene Thermal Maximum (HTM), a warm and dry climate mode dated to about 8000-5000 cal yr BP in the region (17).

Regional factors like climate, nutrient concentration, anthropogenic disturbances and physical oceanographic conditions like changes in current patterns can influence diatom species (18) and render them as a useful probe to study environment induced adaptations over long timescales. So far, documented viability of diatom resting spores from marine sediments is in the order of a century, when older studies with less good time control are excluded (7). In the open Baltic Proper, high resting spore abundance is found during three time slots: the HTM (ca. 8000-5000 cal yr BP) (17), Medieval Climate Anomaly (MCA) (ca. 950-1250 AD) (19) and the last century (20). The abundance of resting spores in sediments from the Baltic Basin are attributed to high primary production and eutrophication (20, 21)and also coincides with high temperatures (22) and high surface water salinities, at least during the Holocene Thermal Maximum (23).

Existence of millenia old *Chaetoceros* resting spores in the Baltic Sea

A number of phytoplanktons such as *Chaetoceros* form resting stages as part of their life cycle and the resting cells overwinter in the sediment as seed stock for the following generation (24, 25). Thus, resting spores form aquatic seed banks equivalent to seed banks of terrestrial plants (7, 26). Resting stages in the sediment are of ecological and paleoecological importance as they can be revived when exposed to suitable environmental conditions and are a source of genetic material for microevolutionary studies (7). Globally, *Chaetoceros* is the most abundant and diverse (58 *Chaetoceros* species are listed only in the brackish Baltic Sea) marine planktic diatom genus and plays a major role in marine primary production (27-29) (Fig. 1). *Chaetoceros* are found due to its dissolution-resistance, mainly as resting spores throughout the stratigraphy from early Littorina Sea stage about 7500 years ago till date (19). The distribution of the species in the Baltic Basin correlates with the salinity gradient showing higher diversity in the more marine Kattegatt and Danish straits (55 taxa), 30-28 taxa in the Arkona Basin and Southern Baltic Proper and 8 to 13 taxa in the brackish central Baltic Proper and in the almost freshwater Bothnian Bay (27); Fig. 2). Resting spore formation in diatoms is an effective strategy to survive periods of stress by leaving the plankton environment, and is believed to have enabled diatoms to withstand the catastrophic event causing mass extinction during end of Cretaceous period (30). An abundance of resting spores in sediments suggests a high number of vegetative cells during high productivity in surface waters (31), or by a sudden environmental change (32) e.g. by nutrient depletion at the termination of a phytoplankton bloom. Thus, the concentration of resting spores alone can give an indication of sudden environmental changes.

Results

Resting spore concentration across evolutionary timescales

Here, we investigated the viability and longevity of germination of the resting spores of a *Chaetoceros* sp. from sediment samples from three time intervals from the Landsort Deep which is the deepest (459 m) and the anoxic region of the Baltic Sea. This regions was selected with an expectation that the resting spores would be well preserved in these environmental conditions. First, we estimated the age of our sediment samples. The radiocarbon dating showed that our sediments samples were from three distinct time intervals from present time (0 years) to 7200 year BP (with the calibrated calendar year BP ages, see Table S1). The sediment from the short gravity core M0063I with an anticipated age of the last century could be assigned an age of ca. 1940-1935 (i.e. ~ 0 - 80 years) at 63 cm, based on the stratigraphic time markers identified (Fig. 3).The sediment from MCA age (M0063C 5.42-6.32 meters composite depth (mcd)) was dated to ca. 1300-1000 cal yr BP and the oldest sediment of HTM age (M0063D 21.63-26.05 mcd) was dated to ca. 7200-6000 cal yr BP. It was possible to estimate the calendar years age for all the levels analyzed

with the exception of 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 S2).

We also reconstructed the resting spore concentrations of the *Chaetoceros* spp. and observed that the highest concentration (~200 million resting spores per gram dry weight of sediment at 26 mcd) in the sediments deposited during the HTM, after which concentrations decreased gradually (Fig. 4). Preservation of diatom frustules 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 sequence, but concentrations were relatively low. Due to the preservation issues, reconstructed concentrations might not be accurate in this sequence. During the MCA age, resting spore concentrations were lower (~23 million around 6 mcd) than observed during HTM age, following which concentrations decreased again. The most recently deposited sediments revealed a pronounced increase in *Chaetoceros* sp. resting spore concentration (~146 million/gdw) as high as found during the HTM age. The resting spore formation is often a response to nutrient depletion at the termination of a phytoplankton bloom and their occurrence often coincides with high productivity in surface waters (28=29) which suggests accumulation of high nutrient concentration possibly due to anthropogenic effects.

Germination of recent and millenia old resting spores

Next, we revived the ancient and recent resting spores. Currently, only a century old resting spores have been revived (33). A modified protocol (see Methods) was used to revive the recent (~0-80 cal years) and ancient (1300-1000 cal. yr BP and ~7200-6000 cal yr BP)) resting spores. The environmental conditions were recreated to germinate the resting spores (Fig. 5a) from the three ages which included reconstructing the salinity and low temperature conditions during the HTM. The resting spores from all three ages germinated in ~ 2 to 3 days. The germination of one *Chaetoceros* species was dominant and consistent between 16 – 31°C (Fig. 5b). The resting spores germinated (2.1 and 2.6 div d-t, respectively) at temperatures ranging from 16 - 31°C. The number of resting spores which germinated from the three time periods (0-80 years (last century), 1300-1000 cal yr BP (MCA) and ~7200-6000 cal yr BP (HTM) varied. A greater number of the younger (~0-80 calendar years and 1300-1000 cal. yr BP) resting spores germinated as compared to the oldest (~7200-6000 cal yr BP) resting spores. Three to four *Chaetoceros* species germinated in several layers, but the presence of one *Chaetoceros* species was dominant at the salinity (~11-11.5) and temperature (<15-16°C) conditions provided. Nine populations of the resting spores isolated from the last century could be grown in cultures. The old spores (~1300-1000 and ~7200-6000 cal yr BP) of the MCA and HTM age germinated but could not be grown in cultures.

Extraction of Holocene DNA

Here we revived and cultured the resting spores from sediments from the last century to ~7200 cal. yrs BP by recreating the ancient salinity and temperature conditions.

We extracted high quality DNA with concentrations ranging from ~100 to 235ng/ul with a A260/A280 ratio of ~1.7 to 2 from the cultures of the germinated resting spores of the last century (~ 0 - 80 years) as shown in the gel (Fig. 5c). Single cell Chelex® DNA extraction on the older spores (~1300-1000 cal yr (MCA age)) with a A260/A280 ratio of ~1.7 to 2 was done. We amplified six primer pair combinations from rRNA (SSU) molecules and chloroplast genes (*rbcL*) from three cultured population (Tables S3-S4) (Fig. 5b). After 30 cycles of PCR, partial sequences for chloroplast genes, rbcL (~ 403- 980 bp) and rRNA molecules, SSU (406–550 bp) were obtained by Sanger sequencing for three sediment revived resting spore populations of *Chaetoceros* (Fig. 5d), the sequences for the remaining genes were not obtained (Table S4). Sequence alignment and blast results of the nuclear ribosomal RNA (small subunit [SSU]) confirmed that the sequences were from the *Chaetoceros* species. Blast searches using the new sequences resulted in matches consistent with the genus-level morphological identifications of our specimens. Our validation of methods across populations showed no differences in the overall sequence alignments.

Identification of the Chaetoceros muelleri species

Furthermore, there have been attempts to use the resting spores for paleoenvironmental reconstructions by assigning them to species, but most of the resting spores are hard to classify (34). This has severely limited our understanding of paleoproductivity and the effect of environmental change on species. However, revival (germination) of resting spores can provide better biomarkers to identify the species of the spores. Therefore, we explored the possibility of reviving resting spores of *Chaetoceros* in order to classify the spores into species. We identified the germinated *Chaetoceros* resting spores that we were able to culture as *Chaetoceros muelleri* Lemmermann (Fig. 6). *Chaetoceros muelleri* unlike other *Chaetoceros* taxa is a solitary living species with their vegetative cells characterized by a convex or flat valve surface (35) (Fig. 6A-E; Johansen and Rushforth 1985), and their resting spores as smooth with one valve bowed and the other protruded and truncated (34). The above characteristics also fit with the resting spores found in the culture (Fig. 6F-J). The taxa has been reported from brackish waters in Europe and North America (35,36). In the Checklist of Baltic Sea Phytoplankton Species *C. muelleri* is recorded in nearly all subareas and considered a warm water species found in low salinity and mainly eutrophic waters (13). Further, *C. muelleri* was one of the resting spores identified in sediments deposited in the Littorina Sea from the southern Baltic Proper (34).

Discussion

Assuming physiological salt concentrations, neutral pH and a temperature of 15 °C, it would take about 100,000 years for hydrolytic damage to destroy all DNA that could reasonably be retrieved (37, 38). Some environmental conditions, such as lower temperatures (39), will extend this time limit, whereas other conditions like oxidation, extreme pH values will reduce it. Other factors, such as rapid desiccation and high salt concentrations, may also prolong DNA survival (40). Therefore, we hypothesized that high quality DNA can be extracted from the sediment revived *Chaetoceros* spores given the high salinity (12-13) during the HTM (7200-6000 cal. yr BP), the high sedimentation rate (quick burial of spores) and the anoxic condition in the Landsort Deep site in the Baltic Sea.

Ancient DNA research has progressed immensely due to the introduction of a new generation of DNA sequencing technologies (41). However, the quality of DNA with respect to the damage caused with time contain inherent problems, particularly with regards to the generation of authentic and useful data and reproducibility. The solution currently advocates reducing contamination and artefacts by adopting certain criteria for authentication (42). On the contrary, resurrection ecologists can bring back to life viable dormant propagules of ancient aquatic organisms that allow phenotypic or genomic characterization rather than piecing together fragmentary fossil DNA. Resurrected organisms provide exceptional opportunities to study evolutionary processes, are a potential source of extinct species or lineages, and can be used to test paleo-proxies of environmental change (9,10).

By reviving sediment derived resting spores and DNA extraction we have demonstrated that the sediment *Chaetoceros muelleri* resting spores is a unique system for understanding species adaption and evolution across geological timescales and address questions about the impact of human-induced perturbations on life on Earth. Previous studies on persistence of diatoms in marine sediments have reported germination of viable resting stages from sediment layers ~30–40 cm below the sediment surface. The sediments in the previous studies were estimated to be 175–275 years old based on the sedimentation rates of 1.2–1.5 mm per year (43). In previous studies a century old sediments were dated (33). In our study we used dated thousands of years old (~7200-1000 cal yr BP) dated samples to show that it is possible to revive and also extract DNA from millennia old resting spores. Reliable viability, germination, and accurate age data beyond century long survival times of resting spores remains unknown (2). Our study fills this gap and provides the ages of several millenia old (up to 7200 cal. yrs BP) resting spores obtained from sediments whose

ages were determined by radiocarbon dating, and also the viability and germination record and the quality of DNA obtained from the sediment revived resting spores thus contributing to further understanding within this field. This study showed that germination of recent and millenia old resting spores makes species identification possible thus making it possible to do paleoenvironmental reconstructions.

The isolation of high quality DNA from the resting spores of *Chaetoceros* species as old as ~1000-1300 years (MCA) reveals that the DNA in the sediment revived resting spores are well-preserved and hence presents itself as an excellent model system for tracing the evolutionary history of species. Previous studies especially in humans has revealed that despite treatment of the laboratory equipment with bleach, UV irradiation of the entire facility, protective clothing and face shields and other routine precautions, contamination is a continuous threat, if for no other reason than because the specimens themselves might be contaminated with modern DNA (44). Hence, the availability of high quality DNA from a system preserved so well is like a dream come true and provides immense potential for future microevolutionary studies. This gives the opportunity to get more markers, a complete genome, make comparisons of populations across temporal and spatial scales both genetically but also experimentally.

We found that DNA from single cells of the older spores (~ 1000-1300 cal yrs BP) could be extracted but the cells could not be grown in cultures. It is possible that the older spores had the ability to survive but were unable to reproduce. The germination rates did vary between the different ages, which also suggests that just to remain viable over thousands of years has been a challenge for the species. So, the genetic and hence the physiological changes due to external anthropogenic effects such as eutrophication and chemical pollution and subsequent environmental changes over a thousands years could have affected its ability to reproduce. Thus, these findings will help us to study the differences in mutations and gene expression in the recent versus the old individuals to be able to understand this phenomenon. Amplification and sequencing of additional nuclear and chloroplast markers and, housekeeping genes like TBP (encoding the TATA box-binding protein) and EFL (encoding the translation elongation factor-like protein) and psbA (D1 protein of the photosystem II reaction centre core complex) can provide us more insights into this phenomenon because the expression of both TBP and EFL are considered to be stable under light-dark cycle and psbA is a stress response gene.

Our study demonstrated the (i) successful germination of recent (~0-80 years) and ancient resting spores (~7200-1000 cal yrs BP) (Fig. 5a,b), (ii) DNA from cultures of the recent spores (~0-80 years) and the single cells of millenia old (~1300-1000 cal yr BP) sediment revived resting spores, and (iii) a method to identify the species from resting spores useful in the fields of microevolutionary, micropaleontology and paleoecology studies. Furthermore, this study used thousands of years old dated sediments as opposed to previuos studies where only a hundred year old sediments were dated (33). Hence, the potential reassembly of complete ancient genomes seems imminent. Adequate comprehensive studies of population genetic structure in phytoplankton species along environmental gradients like salinity combined with experimental data is lacking. A previous study has shown that Baltic Sea populations displayed reduced genetic diversity compared to North Sea populations. Growth optima of low salinity isolates were significantly lower than those of strains from higher native salinities, indicating local salinity adaptation (45). Here we can examine the genetic diversity in *Chaetoceros* populations from sediments drilled in the Landsort Deep across evolutionary timescales: ca. 80 years (last century), ca. 1300-1000 cal yr BP (MCA) and ca. 7200-6000 cal yr BP (HTM) ages to trace the evolutionary changes in these species. Future studies can include identification of genetic markers and differences in gene expression levels to determine the changes in the population genetic structure across environmental gradients over time in *Chaetoceros* populations revived from sediment. Population genomics studies using DNA data obtained from modern DNA technologies and single cell genomics together with experimental

studies can be used to understand the evolutionary changes in the species due to environmental change or climate change or anthropogenic effects and to gain insights into the mechanisms of molecular evolution.

Methods

Sampling

The Integrated Ocean Drilling Program (IODP) Expedition 347 drilled the Baltic Proper from R/V *Greatship Manisha* during 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 was drilled during this expedition (Fig. 2). Five holes (M0063A to M0063E) were drilled using an advanced piston corer down to a diamicton (poorly sorted sandy sediment with pebbles which are glacial deposit called till) was reached at about 92 meters composite depth (mcd) (45, 46). The sediments were stored in the dark in a temperature controlled room at 4°C. All Expedition 347 cores were split and sub-sampled during the onshore science party at IODP Core Repository in Bremen, Germany, January to February 2014. Sediment samples used for revival experiments were requested from holes C and D in March 2015. 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, N58°37.34', E18°15.29') and 63 cm (named M0063I, N58°37.35', E18°15.25') respectively were retrieved and immediately subsampled in 1-cm slices and stored in the dark in a fridge at 4°C.

Lithology and sample selection

Site M0063 is divided into seven lithostratigraphic units (46,47) (very briefly described as; below 92 mcd diamicton (till), 92 – 32 mcd laminated clays on cm-scale which gradually change to mixed clays in the upper part (Baltic Ice Lake and Yoldia Sea), 32 – 26 mcd homogeneous clays with sulphide banding (Ancylus Lake), 26 – 0 mcd organic rich clays with more or less pronounced laminaes on mm-scale (Littorina Sea and Post Littorina Sea).

Based on previous studies (15) and results from the onshore science party (46, 47), sediments from three sequences of different age with anticipated high spore abundance were selected to trace the genetic diversity and the evolutionary changes in the *Chaetoceros* populations: the last century (M0063I 0-63 cm), MCA (M0063C 5.42-6.32 mcd) and the HTM (M0063D 21.63-26.05 mcd).

Resting spore concentration

Sediment samples from the upper 30 m (mcd) at 0.5 m intervals from hole M0063D were freeze-dried, and a known weight of sediment was subsampled for the preparation of samples for diatom analysis. Diatom suspensions were prepared according to standard procedures (48), and microspheres were added to allow for diatom concentration reconstructions following Battarbee and Kneen (1982) (49). A total of 49 samples were analyzed for diatoms using light microscopy at 1000x magnification and immersion oil. *Chaetoceros* resting spores were not identified to species level. Concentrations of *Chaetoceros* resting spores were calculated according to Battarbee and Kneen (1982) (49), and expressed in numbers of valves per gram dry weight (gdw).

Dating and age modelling

The youngest sediment (the two gravity cores M0063H and M0063I), was dated using stratigraphic time markers (50). In order to identify stratigraphic time markers, mercury (Hg) and artificial radionuclide (^{137}Cs and ^{241}Am) measurements on M0063H have been carried out. Core correlation of M0063H and M0063I is based on characteristic features of the mercury downcore profiles. Hg and ^{137}Cs were normalized to bulk organic carbon (TOC) in order to eliminate the dilution effect of the massively occurring manganese-carbonate layers.

The other two selected time intervals corresponding to the MCA (M0063C) and the HTM (M0063D) were dated by radiocarbon. Six bulk sediment samples have been dated by radiocarbon accelerator mass spectrometry (AMS) at Beta Analytic, USA.

In order to assign a calendar year age to each levels analyzed an age-depth modeling was performed using the age-depth modeling software CLAM version 2.2 (51) with 2000 iterations and a custom-built calibration curve based on the *IntCal13* calibration dataset (52) with a mean and standard deviation of 900 and 500 ^{14}C years respectively as given by Obrochta et al. (2017) (53). The sediment surface is assumed to be modern (i.e. 2013 the year of the coring).

Single resting spore isolation

Sediment samples from the last century (~0-80 years), MCA age (1300-1000 cal yr BP) and HTM age (7200 - 6000 cal yr BP) were isolated. Each sample was diluted with ~ 1ml of sterile ddH_2O in a 30mm x 15mm petri dish and a drop of the solution was placed on a concave microscope slide. Individual cells were isolated through manual suction using 20–40 µl drawn-out disposable pipettes and examined on a concave microscopic slide under an inverted microscope with a 10x objective and a WF40x eyepiece with a total magnification of 400x. This isolation procedure was modified from Throndsen (1978) (54). The isolated cell with associated contaminants was transferred to a new ddH_2O water droplet. This isolation and transfer was repeated 2–5 times to remove any contaminants. Individual cells were then isolated for the final time and transferred to a 30mm x 15mm petri dish containing artificial seawater medium.

Germination of the resting spores

The resting spores from the last century, MCA and HTM ages were germinated in 30mm x 15mm petri dishes containing artificial seawater medium (Tropic marine) with salinities ranging from ~10.5 to ~11 at temperatures ranging between 15 to 18°C.

Growing the unialgal Chaetoceros cultures

A unialgal cultures of *Chaetoceros* from revived resting spores from the last century (~ 0 - 80 years) were established from an inoculum from the petri dish which was transferred to a 50 ml angled neck Nunc® EasY Flasks™ with vent caps obtained from Sigma-Aldrich. The flasks were filled with a stock solution of the Guillard's (F/2) Marine Water Enrichment Solution (with silica) and grown in growth chambers with controlled temperature (15 -18°C) and a 12:12 light-dark regime. The stock solution was prepared by adding 20 ml of the Guillard's F/2 medium in 1L of artificial seawater. The light intensity was 125, $uE\ m^{-2}\ s^{-1}$ and was provided by fluorescent lamps. The cultures were subcultured every two weeks to maintain the purity of the culture.

DNA isolation from diatom cultures

Total DNA was extracted from each *Chaetoceros* culture (based on revived spores from last century) as described below. 25 ml of the cell cultures were centrifuged at 2500 rpm for 10 min in a 50 ml Falcon tubes, 20 ml of the supernatant was discarded using a syringe. The remaining 5 ml were centrifuged again at 2500 rpm for 5 min. 4 ml of the supernatant was discarded. The remaining 1 ml was spun for 2 minutes. The supernatant was discarded. Genomic DNA was extracted using a modified chloroform method with the addition of cetyltrimethylammonium bromide (CTAB) as describe by Zuccarello and Lokhorst (2005) (55). The culture pellet was grinded with 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 (100mg/ml) and 5 µl Proteinase K (20 mg/ml) and the tubes were incubated at 55-60°C for 30 min. Equal volume of chloroform:isoamyl alchohol (24:1) was added and mixed. The tubes were then spun at full speed (12,000 g) for 5-10 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 (full speed). The DNA pellet was washed in 500 µl of 70% ethanol and the tubes were spun for 5 mins. The supernatant was poured out and the DNA pellet was air-dried. The DNA pellet was suspended in 50 µl of 0.1 X (0.1mM 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.

DNA isolation from single Chaetoceros cells

Single cell Chelex® DNA extraction was performed on spores (~1300-1000 cal. yrs BP) which were obtained from the sediments of MCA age by using the Chelex method. For DNA extraction, Chelex-stored samples were incubated for 20 min at 95°C. They were then vortexed for 15s and centrifuged for 15 s at 12,000 g.

PCR and sequencing

For the PCR reaction, 25 µL of reaction mixture contained as a final concentration, the two primers at 15 pmole each; 5–20 ng DNA template; 0.2 mM each dATP, dCTP, dGTP and dTTP; 2.5 µL of 10×PCR buffer for Blend Taq ($MgCl_2$ concentration); and 0.1 units of Blend Taq polymerase (Thermo Scientific). The PCR condition was as follows: an initial step at 94°C for 2 min followed by 30 cycles with a denature temperature of 94°C for 30 s, an annealing temperature of 53°C for 30 s and an extension temperature of 72°C for 1.5 min. DNA was amplified from individual resting spores of *Chaetoceros* sp. which have been detected from the sediments of the last century. The PCR was assessed by visualizing the products on 1.5% agarose gel. Six primer pairs from nuclear ribosomal RNA (SSU) and chloroplast DNA (*rbcL*) from three populations were amplified (Table S3). Sanger sequencing was performed on the PCR products (Macrogen inc.) of six nuclear ribosomal RNA (small (SSU) and chloroplast DNA (*rbcL*) markers which was used to amplify *Chaetoceros* species (56) (Table S3).

Analysis of DNA sequences

The sequences were blasted using the nucleotide BLAST tool of NCBI. Sequences were aligned by using Molecular Evolutionary Genetics Analysis version 5 (MEGA5) (Table S4).

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Tables.

Table S1. AMS radiocarbon dates and calibrated ages of sediment material from Expedition 347, Site M0063.

Laboratory ID	Site	Hole	Core	Section	Top (cm)	Bottom (cm)	Half	mcd top	14C Age	error	Calibrated age 2 sigma range median	min	max
Core top								0,00			-63		
Beta 418207	M0063	C	3	2	10	11	W	5,22	1760	30	962	577	1373
Beta 418209	M0063	C	3	2	110	111	W	6,22	1900	30	1301	872	1768
Beta 418041	M0063	D	7	2	85	85,5	W	15,65	4590	30	4385	3690	4966
Beta 418042	M0063	D	8	2	35	36	W	17,29	5120	30	5077	4533	5578
Beta 418043	M0063	D	9	2	79	80	W	20,13	5480	30	5612	5066	6154
Beta 418046	M0063	D	15	1	17	18	W	27,45	7380	30	7604	7040	8099

Table S2. Calibrated ages samples analyzed (italics indicates interpolated age).

Core	Sample ID	Depth mcd	Calibrated age 2 sigma range median	min	max
M0063C	347-39293	5,42	1030	693	1416
M0063C	347-39299	6,02	1233	854	1641
M0063C	347-39300	6,12	1267	864	1703
M0063C	347-39301	6,22	1301	872	1768
M0063C	347-39302	6,32	1335	880	1833
M0063D	347-29645	21,63	6022	5579	6459
M0063D	347-29748	21,83	6076	5653	6499
M0063D	347-29750	23,74	6595	6234	6950
M0063D	347-30184	24,07	6685	6321	7035
M0063D	347-30204	26,05	7223	6760	7640

Table S3. Primers used for PCR and sequencing of rDNA and chloroplast genes in *Chaetoceros muelleri*.

Primers	Orientation	Nucleotide*a* sequence	Target gene	Target species	References
Cy18F150	Forward	GCTAATACATGCATCAATACC	SSU	*Chaetoceros gracilis*	Lee et al. 2013
Dt18F150	Forward	ATACCCGTAGTAATTCTAGAGC	SSU	*Chaetoceros gracilis*	Lee et al. 2013
Dt-rbcL-F56	Forward	AGTGACCGTTACGAATCTGG	rbcL	*Chaetoceros gracilis*	Lee et al. 2013
Dt-rbcL-R1010	Reverse	AGGATCACCTTCTAATTTACC	rbcL	*Chaetoceros gracilis*	Lee et al. 2013
18R560	Reverse	GCTATTGGAGCTGGAATTACC	SSU	*Chaetoceros gracilis*	Lee et al. 2013

Table S4. Sequence results of rRNA (SSU) molecules and chloroplast genes (rbcL).

Population	Marker	Target gene	Length (bp)
3	Dt-rbcL-F56, rbcl	rbcL	980
3	Dt-rbcL-R1010	rbcL	962
1	Cy18F150, SSU	SSU	406
1	18R560, SSU	SSU	410
3	Cy18F150, SSU	SSU	406
3	18R560, SSU	SSU	406
1	Dt18F150, SSU	SSU	550
1	18R560, SSU	SSU	420
2	Dt18F150, SSU	SSU	545
2	18R560, SSU	SSU	540

Figures

Fig. 1.

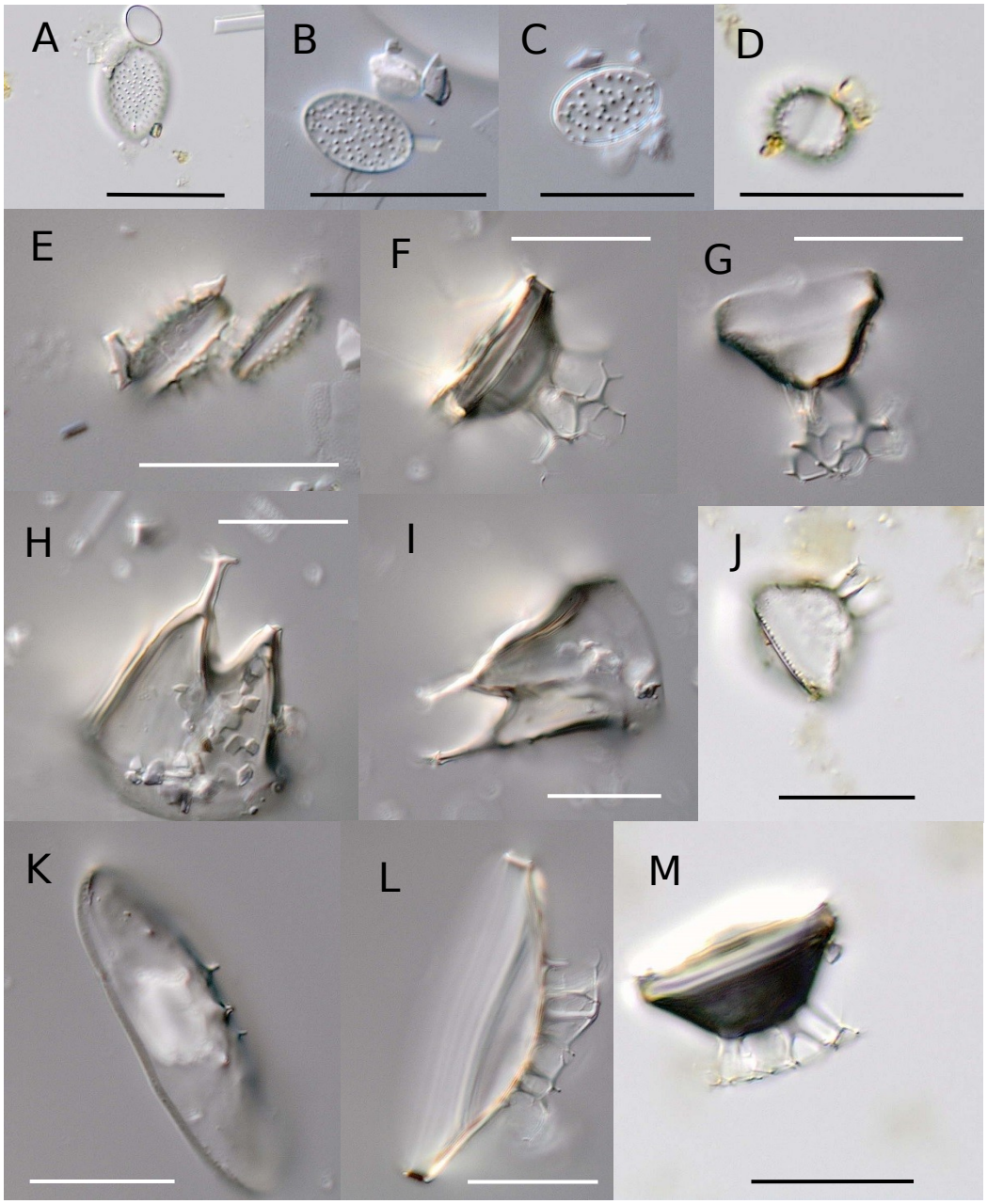


Fig. 1.

Morphologies of selected *Chaetoceros* resting spores from site M0063 Landsort Deep show a diversity of shapes. A-C *Chaetoceros* spp. resting spores in valve view. D-E *Chaetoceros* spp. whole resting cell in girdle view. F-G, L-M girdle view valve of *Chaetoceros* cf. *diadema* resting spore. H-I girdle view valve of *Chaetoceros mitra* resting spore. J-K *Chaetoceros* spp. resting spore. Scale bars 20 microns.

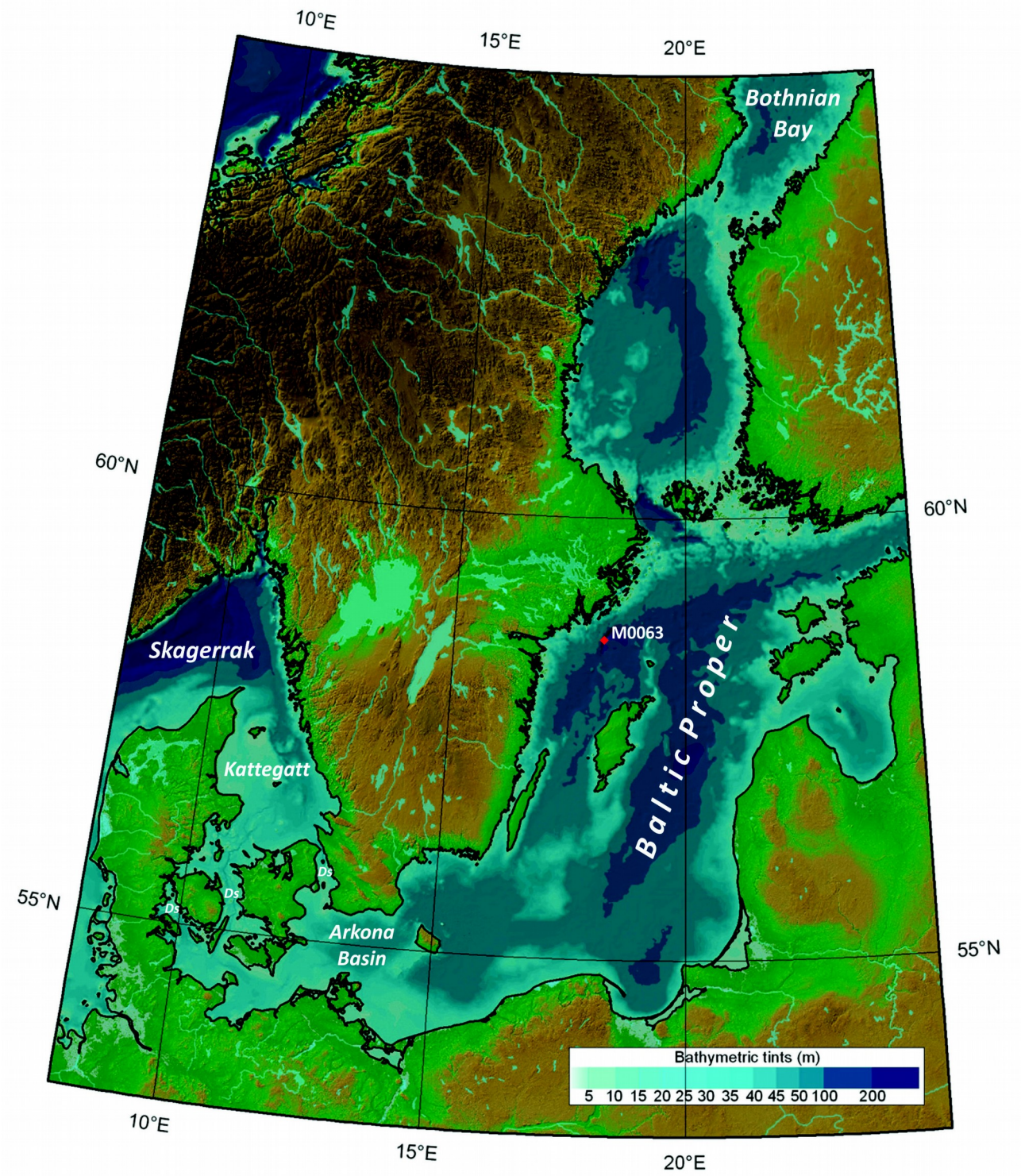


Fig. 2.
Map showing the position of the investigated IODP Expedition 347 site M0063 in the Landsort Deep (58°37.32'N, 18°15.24'E) where a ca 116 m long sediment core was drilled at a water depth of 437 m (Andrén et al. 2015a). Baltic Sea has a long salinity gradient in the surface waters ranging from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

Fig. 3. 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 c. AD 1940 which can be assigned to this depth.

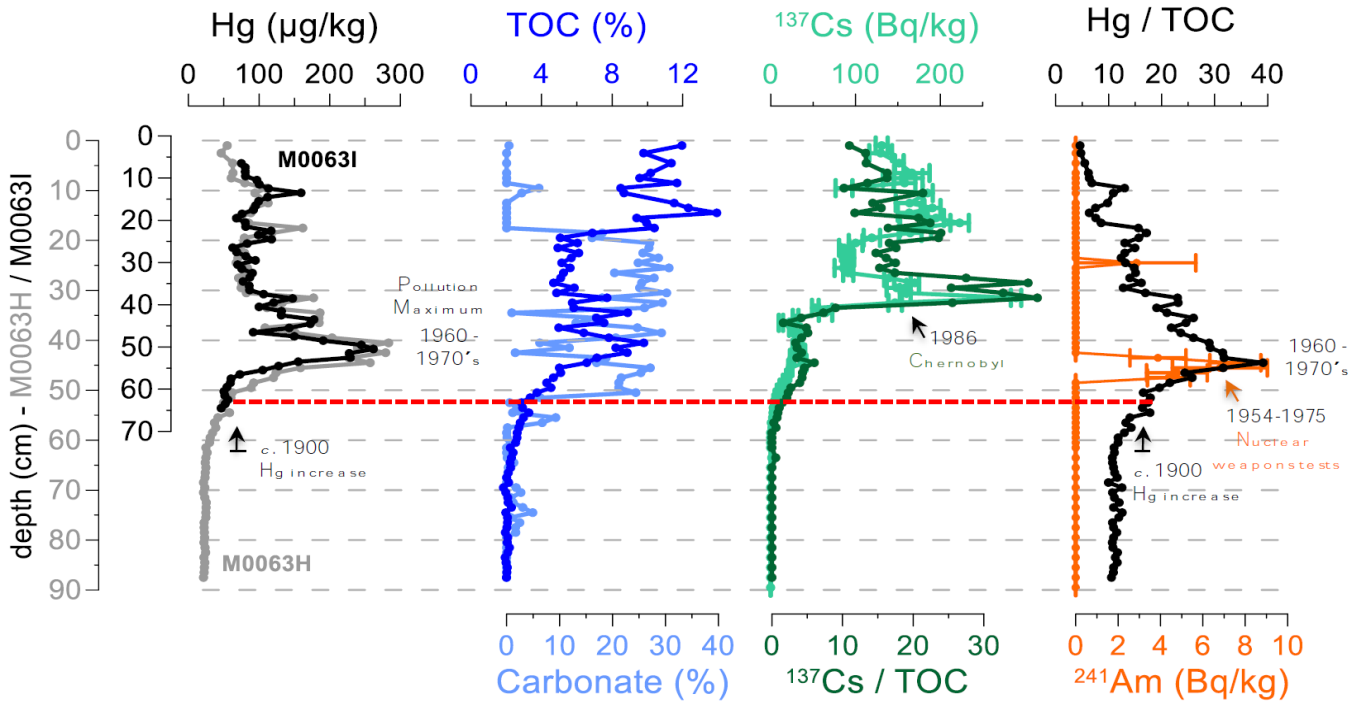


Fig. 4. Lithology and reconstructed *Chaetoceros* spp. resting spore concentrations in the upper 30 meter sediments of IODP Exp. 347 hole M0063D (Landsort Deep).

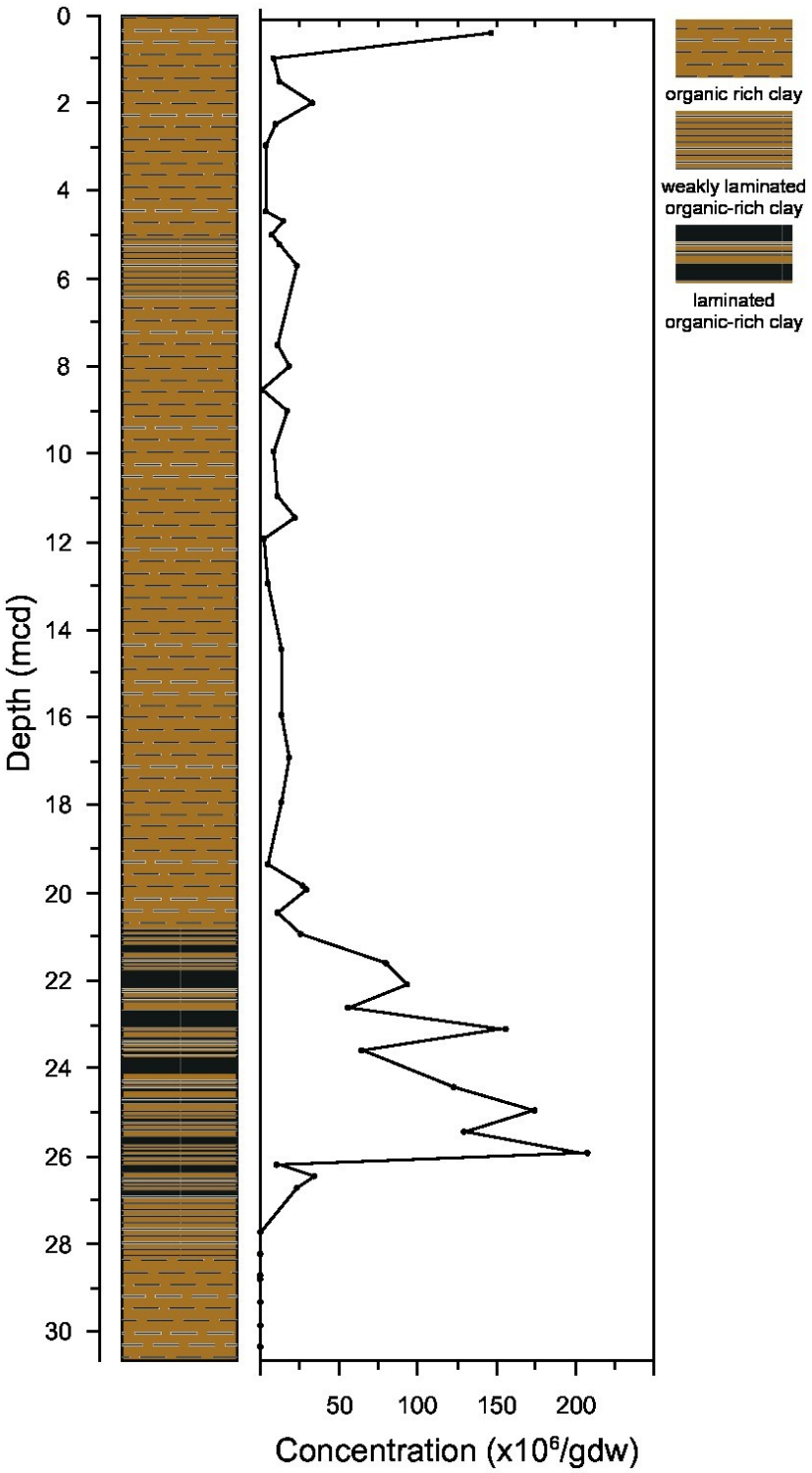
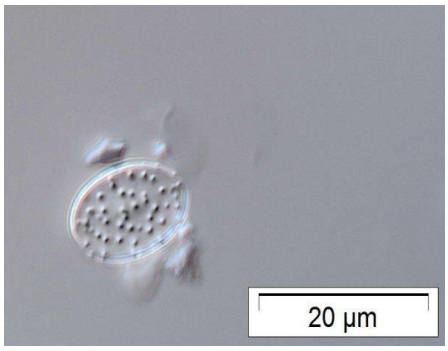


Fig. 5 (A) *Chaetoceros muelleri* resting spores. (B) Germination of resting spores of *Chaetoceros* sp. (C) Agarose gel with DNA from sediment revived resting spores of three populations in lanes 4, 8,9 and 10. (D) Sequences and alignment of the rbcL and SSU rDNA of *Chaetoceros muelleri*.

A



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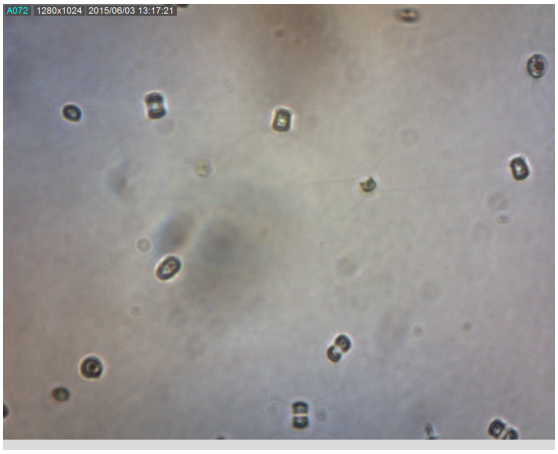

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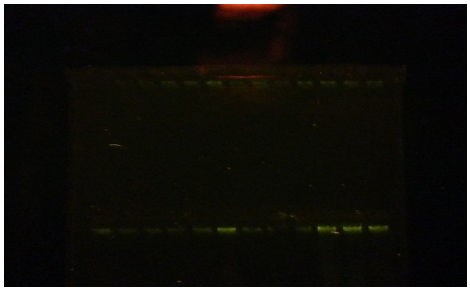

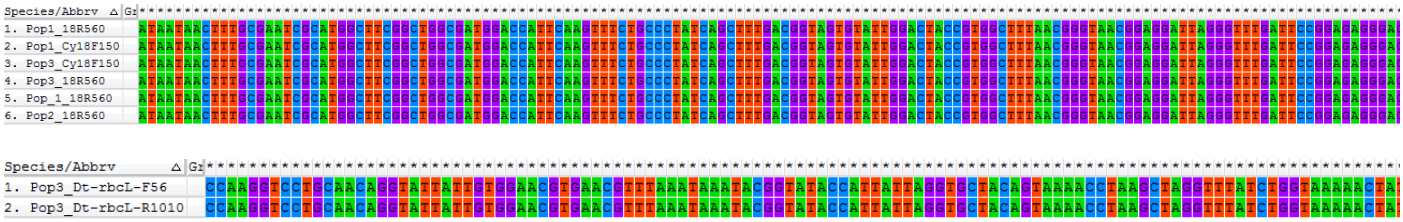

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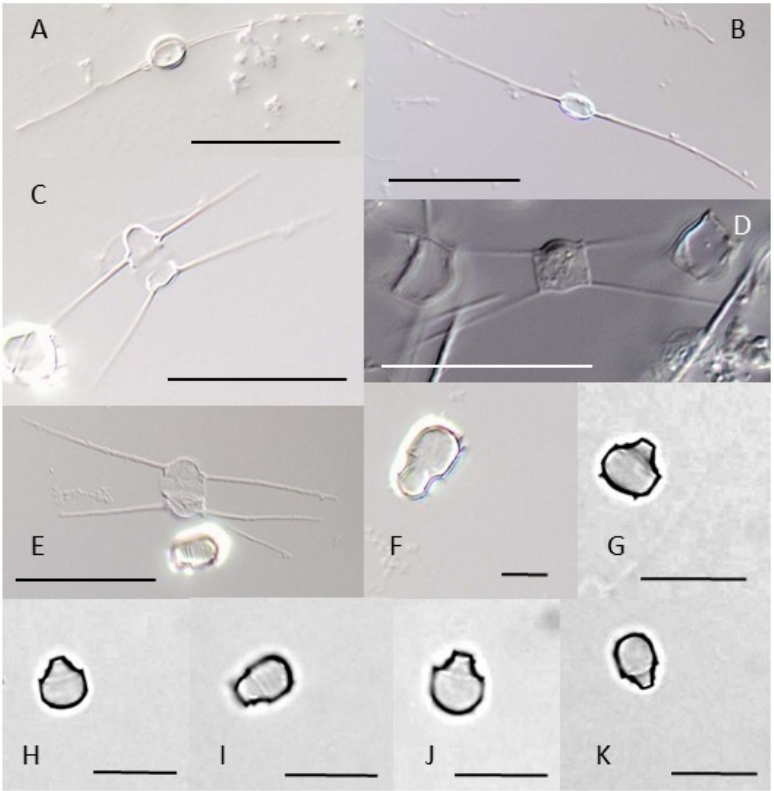

Fig 6. Light micrographs of *Chaetoceros muelleri* Lemmermann in the culture revived from the sediment at 10-11 cm depth. A-B. Smooth solitary cell in valve view. C-E. Cells in valve view showing more or less convex valve shape. F-J. Resting spores in girdle view showing various morphologies. Scale bar A-E 20 microns, F-K 5 microns.