LIMNOLOGY AND OCEANOGRAPHY
Letters

ASLO
Open Access

Limnology and Oceanography Letters 2022

doi: 10.1002/lol2.10291

LETTER

Cross-contamination risks in sediment-based resurrection studies of phytoplankton

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Scientific Significance Statement

To cope with adverse conditions in variable environments, organisms may have evolved long-term dormancy traits. A wide range of aquatic microorganisms maintains a sediment seed bank that might reveal ecological and evolutionary characteristics of populations. However, substantial methodological and technical challenges are associated with retrieving and reviving samples. To date, age determination of revived organisms relies on radiometric dating of bulk sediment or associated fossils rather than the age determination of eggs, spores, or resting stages themselves. Here we show that due to contamination from surface sediments, this approach could be flawed, leading to age artifacts biased toward longer survival estimates in more abundant taxa. Our experimental observation highlights an urgent need for standardized sediment processing practices and improved contamination controls in the field of sediment-based resurrection biology.

Abstract

Resurrection studies can answer some fundamental questions in aquatic ecology and evolutionary biology. For phytoplankton resting stages, longevity of thousands to millions of years has recently been reported. However, contamination during sediment sampling could distort these estimates, and this risk has not been systematically evaluated. Here we used 4.5 μm diameter microspheres to quantify contamination while reviving the resting stages of seven abundant estuarine diatom and cyanobacterial taxa. We observed a sharp decline in resting stages abundance from 10^6 (g wet sediment)$^{-1}$ at the surface to < 0.8 (g wet sediment)$^{-1}$ at 12.5 cm depth. Added microspheres ($\sim 4.5 \times 10^7$ cm^{-2}) were translocated even deeper down the sediment and could well explain the vertical distributions and abundances of revived cells. Without this control, we could have claimed to have revived seven multi-decades to centennial-old taxa. Our findings suggest that improved contamination controls are needed for sediment core sampling of rare cells, microfossils, or DNA molecules.

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Associate editor: Peter R Leavitt

Author Contribution Statement: B.A., K.R., K.J., O.B., and H.L.F. identified the research question and designed the study. B.A., H.L.F., O.B., and O.K. collected field samples. H.L.F. led the geological analysis and constructed the age model, while B.A. and O.K. carried out the biological measurements. B.A. developed the microsphere contamination protocol. B.A. analyzed the data and drafted the first version of the manuscript.

Data Availability Statement: All data and metadata included in this manuscript are accessible through the Swedish National Data Service (https://snd.gu.se/en, doi: 10.5878/9y59-7a50).

Additional Supporting Information may be found in the online version of this article.

The resurrection of dormant organisms can facilitate studies of ecological and evolutionary processes on time scales not feasible in experimental studies (Burge et al. 2018; Ellegaard et al. 2018). To this end, sediments in aquatic environments may contain a viable historical record of zooplankton eggs (Hairston Jr et al. 1995; Kerfoot et al. 1999; Derry et al. 2010), microalgae (Andersen and Keafer 1987; McQuoid et al. 2002), bacteria (Livingstone and Jaworski 1980; Morono et al. 2020), and viruses (Cai et al. 2019). Reports of the longevity of resting stages of phytoplankton range from a year (Garrison 1979), to decades (Stockner and Lund 1970; Livingstone and Jaworski 1980; McQuoid et al. 2002), centuries (Härnström et al. 2011; Lundholm et al. 2011), millennia (Sanyal et al. 2022), and up to 16 million years for a cyanobacterium (Morono et al. 2020). However, dating individual resting stage cells is not yet technologically possible, and age determination relies on radiometric dating of bulk sediment, or co-occurring macro/micro-fossils from the same sediment layers (Ellegaard et al. 2018). Furthermore, unlike plant seeds (Beal 1885; Telewski and Zeevaart 2002; Sallon et al. 2008), direct longevity of phytoplankton resting stages has not been experimentally verified beyond a decade (Lewis et al. 1999). Given the wide range of reported survival times and lack of direct experimental support of survival times, longevity estimates deriving from the position of cells in sediment cores should be interpreted cautiously.

Sediment core samplings are vulnerable to contamination since contemporary cells populate the sediment surface at high concentrations. In resurrection studies, in situ translocation of surface cells due to physical forces or bioturbation has previously been controlled for via vertical profiles of anthropogenic ^{137}Cs (Appleby 1998, 2001), deposition of short-lived ^{234}Th isotopes (Schmidt et al. 2007), or X-ray imaging (Ribeiro et al. 2011; Ellegaard et al. 2013). However, these methods are insensitive to small amounts of contamination during the retrieval and processing of the sediment cores. Cores are mainly retrieved using gravity corers deployed from ships (Ellegaard et al. 2013), although SCUBA or ice-based recovery has also been used for lake sampling (Cáceres 1998; Pedersen et al. 2016). The extraction inevitably causes smearing of surface sediment along the core lining and may cause fissures or release of trapped gas. The sectioning of the core into individual depth segments involves further contamination risk. Although researchers in the field generally acknowledge the risks of contamination, mitigation strategy typically relies on excluding potentially contaminated sediment fractions (Hairston Jr et al. 1995; Cáceres 1998; Ellegaard et al. 2013). Absolute rates of contamination during core processing have rarely been quantified.

Gåsfjärden is a semi-enclosed inlet in the Baltic Sea that we have previously used as a geological model system to study past environmental changes (Ning et al. 2016*a*,*b*, 2018). Changes in diatom microfossil composition appear to correlate with copper mining activity from the adjacent Solstad copper mine during the past 200 years (Ning et al. 2018). Recently, we documented the evolution of elevated copper tolerance in contemporary populations of the abundant diatom *Skeletonema marinoi* (Andersson et al. 2020). The original aim of the present study was to employ a resurrection approach to track the origin of this evolutionary response. To ensure that contamination did not cause age artifacts in the resurrection of the resting stages, we resurrected coastal phytoplankton while quantifying vertical contamination of surface sediment to deeper strata. We employed a fluorescence microsphere-based method to quantify how particles of a similar size to phytoplankton migrated during the processing of cores simultaneously with measuring vertical distributions of the seven most abundant diatom and cyanobacterial taxa. Our quantification of contamination, which is the focus of this article, showed that we could not confidently resurrect ancient resting stages without major contamination from contemporary cells. Although only a case study on phytoplankton, this finding has broad implications for age determination of resurrected organisms or other paleoecological studies targeting rare DNA or microfossil markers.

Materials and methods

Field sampling and core dating

Sediment cores were collected from seasonally hypoxic locations on the Swedish coast of the Baltic Sea. Between 27 and 28 June 2017, two semi-enclosed inlets, Gropviken (GP17; 58°19.92′N, 16°42.35′E) and Gåsfjärden (VG17; 57°34.35′N, 16°34.98′E), were sampled and used for investigation on contemporary resting stages (Andersson et al. 2020). However, the VG17 cores lacked temporal resolution for our resurrection experiments. On the 22nd of September 2020, Gåsfjärden (VG20; 57°34.38′N, 16°35.43′E) was revisited at the exact location sampled in 2011 by Ning et al. (2016*a*), which was 500 m east of VG17. Gropviken was included as a reference site in 2017 since it also has hypoxic and laminated sediments, but has not been exposed to direct mining activity (Karlsson et al. 2010).

Six to ten sediment cores per station and sampling occasion were collected using an 80-cm-long ($\varnothing$ = 9 cm) Gemax corer (OY Kart Ab) deployed from R/V Electra. Cores for radiometric dating and total organic carbon (TOC) and nitrogen (TN) profiles were processed on-site and analyzed as previously described (Verardo et al. 1990; Ning et al. 2016*a*) using gamma spectrometry and elemental analyzer (Costech ECS), respectively. The age of the sediment was estimated based on the constant rate of supply (CRS) model using ^{210}Pb, with ^{137}Cs as a secondary reference (Appleby 1998), and the TOC profiles were used to correlate between previously collected cores and established age models (Ning et al. 2016*a*, 2018). Cores for resting stage enumeration were covered in dark plastic bags, transported intact with overlaying water to the University of Gothenburg via car, and stored at 5°C until

processing. Following the instructions of Ellegaard et al. (2013), great care was used to avoid the smear-contaminated core lining, and the bottom and top of each section, before sampling ~ 30% of the interior. The sectioning of 1-cm sediment slices was performed using the equipment designed for the Gemax corer. All equipment that touched sediment was triple rinsed MilliQ-purified water between sediment slices. The 2017 samples were used for preliminary observations of resting stage abundances and their longevity ex situ (see Supporting Information).

Quantification of contamination using fluorescent microspheres

We quantified contamination during the 2020 sampling. Non-toxic, neutrally charged, yellow-green fluorescing polystyrene microspheres (Thermo Scientific Fluoro-Max) with a density of 1.05 g cm^{-3} were used to track surface sediment contamination in three VG20 cores. Fluorescing microspheres (0.5 μm) have previously been used to control for microbial contamination via surface water cooling in deep-sea drilling (Smith et al. 2000). We used larger (4.5 μm diameter) spheres since they more closely resembled the size of diatom resting stages and were bright enough to minimize the risk of false-positive observations. Spheres were injected into the water headspace of intact cores at a nominal concentration of 4.5×10^7 spheres per cm^2 sediment surface. After 2 weeks, most spheres had settled onto the sediment surface, but for logistic reasons, cores were stored 4 months before sectioning. Bottom-water hypoxia was prevalent at both sample locations, and it is unlikely that the post-sampling storage time impacted on sediment chemistry and viability of the resting stages.

Sub-samples of sediment from every cm in the first 10 cm, every other cm from 10 to 20 cm, the 29th and 49th cm depths were prepared for sphere counting, as described in the Supporting Information. Briefly, organic material was removed via consecutive acid, base, and H_2O_2 treatment, remaining sediment sonicated, and resuspended in a Triton X-100-based solution to reduce aggregation. Microspheres were counted in 1 mL Sedgwick-Rafter chamber at ×200 magnification using a Zeiss Axiovert A1 epifluorescence microscope and filter set 5 (excitation 395–440, beam-splitter at 460, and detection > 470 nm). Two hundred spheres were counted from each sample, or the equivalent of 50 mg of wet sediment if the abundance was too low, with intermittent blanks to control for false positives. Surface contamination was quantified based on changes in the relative abundance of microspheres compared with the surface

$$\text{Surface contamination at depth } i = \frac{\text{microspheres g}^{-1}\text{ at depth } i}{\text{microspheres g}^{-1}\text{ at } 0-1\text{ cm}} \quad (1)$$

Abundance of resting stages of phytoplankton

Viable resting stages of abundant taxa of diatoms and cyanobacteria were enumerated in the VG20 cores using the most probable number (MPN) approach (Jarvis et al. 2010) with the MPN package in R (Ferguson and Ihrie 2019). Sediment was inoculated in f/2 media (Guillard 1975) with 106 μM SiO_2, using 1 mL in 24-well microplates (Falcon) or for 1 g in 20 mL media. The MPN approach works on a dilution-to-extinction principle, and a detailed method description can be found in the supplements. Briefly, dilution series were run in triplicates per sample and modified to encompass the expected resting stage densities at: 0–3 cm depth; four-fold (eight steps from 0.01 g), 3–10 cm depth range; three-fold (eight steps from 0.1 g); 10–50 cm depth; two-fold (eight steps from 0.1 g, and also 1 g). Resting stages were scored as present/absent based on weekly microscope observations during 3 weeks of incubation (Fig. S1) at 16°C and 10–20 μmol photons m^{-2} s^{-1}. Since the MPN method requires cell division to occur for proper scoring, we use this criterion when we refer to viable resting stages. Relative abundance patterns of the resting stages and microspheres were compared graphically to assess the risk of contamination artifacts in the age determination of the phytoplankton. Data, scripts, and metadata are available in Andersson et al. (2022).

Results

Core sampling and dating in 2017

The sedimentation rate was lower than expected at VG17 (0.05 vs. ~ 0.2 cm yr^{-1}; Ning et al. 2018) and about 10 times lower than GP17 (0.77–0.2 cm yr^{-1}). Consequently, the ^{210}Pb CRS age model was well resolved for GP17, but for VG17, the 20th century was contained in the top 6 cm of the core (Fig. S2A–C). VG17 had consistently higher TOC content (mean 7.9% [SD 0.72]) than GP17 (4.4% [0.44]), with a dip from 8% at the surface to 5% in the mid-1900s and returning to 7% again by the mid-180 s (Fig. S2D). The TOC/TN ratio was largely confined between 7.5 and 8 at both stations (Fig. S2E).

In 2017, the surface abundance of contemporary resting stages was dominated by *S. marioni* at between 10^5 and 10^7 (g wet sed)$^{-1}$, followed by one to three orders of magnitude fewer *Thalasiossira baltica* and *Chaetoceros* spp. (Table 1). Abundances of these three taxa decreased significantly with sediment depth and to a similar degree at both stations. *S. marinoi* persisted to the greatest depth, but already at 4–7 cm, the abundance was reduced by four orders of magnitude. Below 12–13 cm depth, there was only one observation of a revived *S. marinoi* in the GP17 core. Radiometric dating of these sediments suggested that the resting stage(s) of *S. marinoi* could have been deposited significantly longer than 140 years ago in VG17, and 60–70 years ago at GP17 (Table 1).

Table 1. Diatom resting stage abundances in two Baltic Sea sediments. Data are based on cores retrieved in 2017, with sediment age computed using a constant rate supply model (see Fig. S2), with linearly extrapolated ages based on sediment accumulation rates shown in italic. *S. marinoi*, *T. baltica*, and *Chaetoceros* spp. resting stage abundances are based on one 10-fold dilution series (seven steps from 1 g) and indicate the ranges between positive and negative growth in dilutions. In surface samples, viability was assessed again after approximately 1, 3, and 4 years of storage (two- to three-fold dilutions, 12 steps, from 0.1 g, see Fig. S3 and supplemental method for more details). Changes in viability are shown as fraction of initial (3 months after field sampling) observation, with the complete data series shown in Fig. S3. n.d., not detected followed by the detection limit in parenthesis.

Sample information				Initial diatom resting stages (viable resting stages [g sed]$^{-1}$)			4-Year survival ex situ
Location	Core	Depth (cm)	Sediment age	*S. Marinoi*	*T. baltica*	*Chaetoceros* spp.	Fraction viable (*S. Marinoi*, *T. baltica*, *Chaetoceros* spp.)
Gåsfjärden	VG1-C	1–2	2003–2010	10^6–10^7	10^3–10^4	10^3–10^4	(10^{-4}, 0.3, 0.1)
		6–7	*1878–1904*	1–10	1–10	n.d.(1)	—
		12–13	*1729–1754*	1–10	n.d.(1)	n.d.(1)	—
		46–48	*854–904*	n.d.(1)	n.d.(1)	n.d.(1)	—
Gropviken	GP2-C	2–4	2014–2015	10^5–10^6	10^4–10^5	10^3–10^4	(n.d., n.d., n.d. [10])
		4–6	2012–2014	10–10^2	1–10	10^3–10^4	—
		36–38	1941–1951	0.5	n.d.(1)	n.d.(1)	—
		46–48	*1890–1901*	n.d.(1)	n.d.(1)	n.d.(1)	—

Ex situ survival of resting stages

Survival of resting stages was monitored for 4 years to determine if *S. marinoi* was more long-lived than other diatoms, as expected from the distribution data. The results showed an opposite trend. In VG17, *T. baltic* and *Chaetoceros* spp. retained between 10% and 30% viability after 4 years compared with 0.1% for *S. marinoi* (Table 1). In GP17, the patterns were similar for the initial 3 years, but no viable diatom resting stages were observed after 4 years of ex situ storage (Fig. S3).

Microsphere-based contamination of surface sediment in 2020

Gåsfjärden was revisited in 2020, at a station 500 m west of VG17. As expected, the sedimentation rate was higher at VG20 (0.23–0.08 cm yr^{-1}) than VG17, which resulted in a better temporal resolution of the 1900s within 20 cm (Fig. 1 vs. Fig. S1). However, the top 3 cm of the surface sediment had been recently oxygenated with signs of small-scale bioturbation (Fig. S4).

Microspheres were added to the VG20 cores and the bulk location could be visualized in intact cores using flash

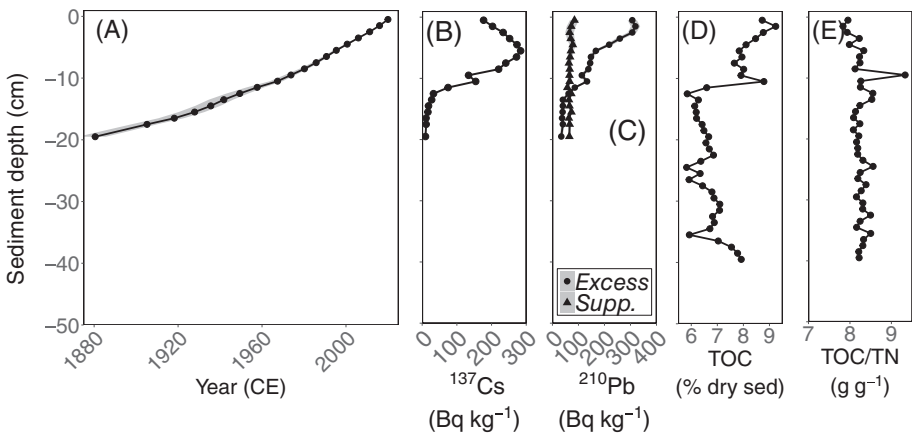

Fig. 1. Core geology data from the 2020 expedition to Gåsfjärden (VG). (**A**) Sediment age computed using the ^{210}Pb constant rate supply (CRS) model, (**B**) ^{137}cesium isotopic concentration, (**C**) supported and excess ^{210}lead isotopic concentration, (**D**) total organic carbon content, and (**E**) total organic carbon to nitrogen ratio. Corresponding data from the 2017 expedition are shown in Fig. S2. Measurements were done on one core (VG20_A) with shaded 95% confidence intervals in (**A**) and (**C**) correspond to measurement accuracy and model uncertainty, respectively.

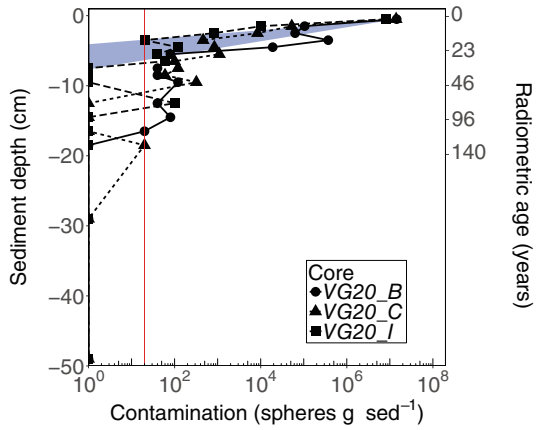

Fig. 2. Microsphere-based quantification of contamination of surface sediment to deeper layers. Data are from three individually processed cores. The blue-shaded area shows expected concentration under a scenario of 1–10% contamination between processed layer/cm. The red vertical line corresponds to the detection limit of microspheres. The right-side axis shows the radiometric determination of the age of the sediment.

photography. In the C and I core, microspheres formed a distinct layer on the surface after 4 months storage, while in the B core, pockets occasionally extended several cm down along the rim (Fig. S4). For reference, we projected that the effects of 1–10% contamination per processed layer (cm) would result in $< 10^{-6}$ fractions of surface contamination below 6 cm depth, while smearing along the rim could result in deeper and more stochastic contamination (Fig. 2). In the top 4 cm, there was a rapid decrease of microspheres in two cores (1.6 and 3.7% retention per cm based on log-linear regression of microsphere density vs. depth for VG20_I and C, respectively [$R^2 > 0.97$]). VG20_B displayed a slower decrease (16% retention per cm, across the top 6 cm [$R^2 = 0.85$]), likely due to a larger number of microspheres having been translocated along the rim during the 4-month storage due to gas-venting (Fig. S4). Microsphere contamination could be detected ($> 2 \times 10^{-6}$ fractions) down to 12.5, 15.5, and 18.5 cm per individual core and became more stochastic with depth (Fig. 2).

Table 2. Absolute concentrations (95% confidence) of microspheres and resting stages in Gåsfjärden 2020 samples. The extinction depth corresponds to the greatest depth where viable resting stages were no longer observed, with the detection limit corresponding to the upper 95% confidence of the most probable number (MPN) prediction. Note that MPN data were not collected on Core VG20_C since its integrity appeared compromised from the core collection.

Taxon	Maximum abundance (g wet sed)$^{-1}$	Maximum abundance depth (cm)	Extinction depth (detection limit [g wet sed]$^{-1}$)
Core VG20-B			
S. marinoi	230,000 (74,000–710,000)	1–2	18–19 (0.86)
T. baltica	21,000 (7000–64,000)	1–2	6–7 (8.6)
Chaetoceros spp.	15,000 (7000–38,000)	1–2	12–13 (8.6)
Fragilariopsis spp.	1,900,000 (540,000-6,900,000)	1–2	12–13 (8.6)
Benthic diatoms	86,000 (29,000-260,000)	0–1	12–13 (8.6)
Dolichospermum spp.	13,000 (5000–33,000)	1–2	12–13 (8.6)
N. spumigena	860 (280–2600)	0–1	12–13 (8.6)
Microspheres	9,700,000	0–1	18–19 (20)
Core VG20-I			
S. marinoi	230,000 (74,000–710,000)	2–3	18–19 (0.86)
T. baltica	3300 (1300–8500)	2–3	12–13 (8.6)
Chaetoceros spp.	3100 (1100–8800)	4–5	12–13 (8.6)
Fragilariopsis spp.	1100,000 (330,000–3,600,000)	1–2	12–13 (8.6)
Benthic diatoms	68,000 (28,000–160,000)	0–1	12–13 (8.6)
Dolichospermum spp.	14,000 (4500–42,000)	0–2	12–13 (8.6)
N. spumigena	1200 (420–3400)	4–5	12–13 (8.6)
Microspheres	8,300,000	0–1	12–13 (20)
Core VG20-C			
All taxa	n.a.	n.a.	n.a.
Microspheres	14,000,000	0–1	18–19 (20)

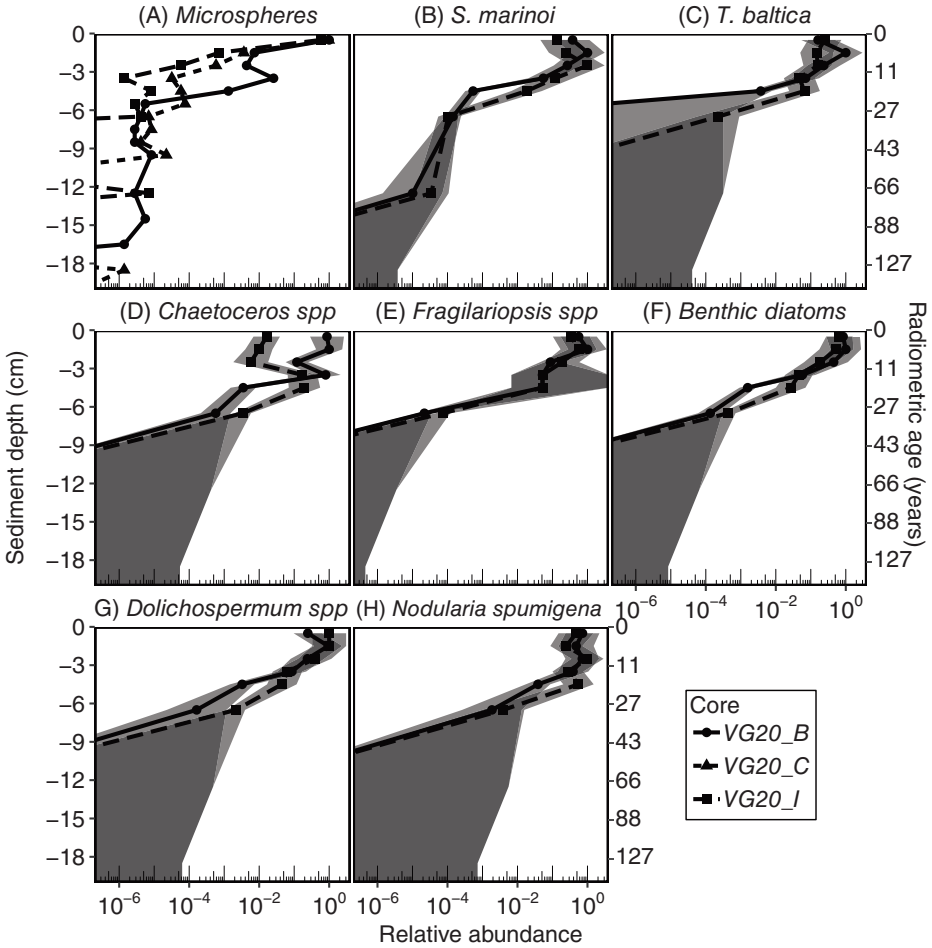

Fig. 3. Vertical distribution of microspheres, phytoplankton, and benthic diatom resting stages in the sediment. Data from cores were collected in the 2020 Gåsfjärden expedition. To facilitate comparison, abundances have been standardized to the highest abundance observation for each panel, with addition of 10^{-10} for zero-count data points. The resting stages (panels B–H) abundances were computed using the most probable number (MPN) approach. For absolute numbers, see Fig. S4. Shaded areas correspond to 95% confidence intervals, with the higher value representing the detection limit in zero observation data points. The right-side axis shows the radiometric determination of the age of the sediment.

Depth distribution of phytoplankton in 2020

Six taxa and one functional group (benthic diatoms) could be consistently quantified using MPN (Table 2). Three were pelagic diatoms, two pelagic cyanobacteria, while benthic diatoms consisted mainly of *Naviculoid* and *Amphoroid* species. The epiphytic *Fragilariopsis* spp. may have been one species, while *Chaetoceros* spp. consisted of many species. Other taxa were observed sporadically, including diatoms (*Melosira* spp.), dinoflagellates (several species), cyanobacteria (*Merismopedia*, *Pseudanabaena*), flagellates, chlorophytes, and small (< 2 μm) single-celled taxa.

Core VG20_C displayed irregular laminations (Fig. S4), possibly due to damage during retrieval, and was not used for MPN. The resting stage abundance profiles in the two other cores were similar across all taxa (Fig. 3 and Fig. S5), with peak abundance at 1–3 cm depth (Table 2). *Fragilariopsis* spp. was the most abundant taxon at 1–2 $\times$ 10^6 (g wet sed)$^{-1}$, followed by *S. marinoi* at 2.3 $\times$ 10^5 (g wet sed)$^{-1}$. Other taxa ranged from 860 (*Nodularia spumigena*) to 8.6 $\times$ 10^4 (g wet sed)$^{-1}$ (benthic diatoms). Only *S. marinoi* resting stages were observed below 6.5 cm depth (Table 2), corresponding to sediment deposited after 1990 $\pm$ 1 yr (Fig. 1). When scaled to peak abundance, the vertical depth profiles of resting stage taxa were similar to each other and the microspheres (Fig. 3). The main difference was that the resting stage profiles had a high subsurface distribution across 1–3 cm depth.

Two observations were potentially inconsistent with a contamination scenario. First, at 12.5 cm, revival was only observed in *S. marinoi*, which is unexpected given that above this depth *Fragilariopsis* spp. made up 41–99% of total resting stage abundance, and *S. marinoi* 1–35% (Fig. S6). However, *S. marinoi* was close to the detection limit at this depth (0.86 [g wet sed]$^{-1}$: Fig. S5) and only observed in combined four well replicates of the MPN. Second, we observed two slow-

growing taxa that emerged during the third week of incubation down to depths of 29 cm in the VG20_B core. One was a benthic cyanobacterium of the genus *Merismopedia*, and the other a solitary, circa 2 μm, coccoid chlorophyte or cyanobacteria. These taxa were not consistently observed above 29 cm depth in VG20_B or in the VG20_I core, potentially due to competitive interactions with more abundant taxa.

Discussion

Sediment-core-based studies of the longevity of phytoplankton resting stages report variable results, ranging from months to millions of years (Lewis et al. 1999; Härnström et al. 2011; Lundholm et al. 2011; Morono et al. 2020; Sanyal et al. 2022). Here we report that revival of what radiometric dating suggested to be > 140-year-old *S. marinoi* resting stages from Gåsfjärden in 2017 could not be reproduced in 2020. Our microsphere contamination control showed that even apparently decade-old diatom or cyanobacteria resting stages were substantially affected by contamination from contemporary surface populations. We also observed a major loss of viability of resting stages stored ex situ during only 4 years. Moreover, the most abundant taxa *S. marinoi*, lost viability faster than other diatoms (Fig. S3), in line with in situ observations from this species in the Mediterranean (Montresor et al. 2013). As resting stages of phytoplankton taxa are present in surface sediments at variable but generally high concentrations (> 10^6 [g sed]$^{-1}$: this study and Chen et al. 2009; Itakura et al. 1997; Sanyal et al. 2022), contamination artifacts might be a problem in resurrection studies and other paleoecological studies based on rare cells, microfossils, or DNA molecules.

Previous studies report multidecadal to millennial survival of the resting stages of the diatoms species *Chaetoceros muelleri* (Sanyal et al. 2022), *S. marinoi* (McQuoid et al. 2002; Härnström et al. 2011), and the cyanobacterium *Dolichospermum* spp. (formerly *Anabaena*) (Livingstone and Jaworski 1980). However, in light of our current findings, these results may need additional confirmation using appropriate contamination controls. After prolonged incubation of 4–6 weeks, we observed emergence of coccoid cyanobacteria and chlorophytes, including *Merismopedia* spp., from sediments down to 29 cm (> 140 years old), with no obvious contamination signatures. This observation suggests that some phytoplankton taxa may indeed be capable to survive for long periods. However, we could not quantify the surface abundance of these taxa due to slow revival and competitive interactions (Fig. S1), and consequently vertical contamination cannot be completely ruled out for these taxa either. We could not quantify the abundance or contamination risk for dinoflagellates cysts because their extended germination window, slow growth, and low abundance make them unsuitable for the MPN method (Fig. S1, and Montresor et al. 2013). Finally, we could not exclude the possibility that low abundant resting stages revived but failed to divide, as reported by Sanyal et al. (2022), required extended incubation times beyond 6 weeks, or chemical stimulants beyond inorganic nutrients and vitamins (Delebecq et al. 2020).

Since we added the microspheres before processing the core, but after transport to the laboratory, our absolute quantification of contamination is likely conservative. Additional undetected contamination most likely occurred during the physical impact of the corer with the sediment surface, winching onto the ship, and during transport. We encourage future studies to also control for this, e.g., via collection of sediment cores from sites prespiked with microspheres or addition of an impact release mechanism to remote core samplers (Tengberg et al. 1995; Bernhard et al. 2009; Pedersen et al. 2016). In addition, we need conclusive evidence of the longevity of resting stages based on ex situ or novel in situ experiments, and also improved insight into physiological mechanisms behind survival in sediments (Lennon and Jones 2011; Ellegaard and Ribeiro 2018). Resting stages could, for example, be buried in nylon mesh bags in sediment and survival rates monitored over time in situ. This would limit predation and contamination while maintaining a natural chemical and microbial environment. Using an analog approach, certain plant seeds have been shown to remain viable in soils for > 120 years (Beal 1885; Telewski and Zeevaart 2002).

To date, few studies have quantitatively evaluated the effects of vertical contamination on sediment-based resurrection. However, findings here suggest that contamination risk increased proportionately to the abundance of contemporary organisms in surface sediments. Consequently, studies focusing on zooplankton, phytoplankton, bacteria, or viruses, and specific species within these taxa, all need to employ different strategies to rule out contamination risks. Population genetic structure and other indirect evidence of changes in resting stage composition can also provide supporting evidence against contamination. There is compelling evidence of evolutionary response reported for zooplankton (Cousyn et al. 2001; Derry et al. 2010; Frisch et al. 2014), suggesting that eggs can survive for many decades. In contrast, analogous genetic and phenotypic population studies on resurrected phytoplankton have reported surprisingly little evolutionary changes over centuries (Härnström et al. 2011; Ribeiro et al. 2011; Lundholm et al. 2017). In conclusion, the findings presented in this study show that contamination can easily cause overestimation of longevity observations in phytoplankton, and that stricter contamination controls are needed to avoid this. Improved contamination controls will provide a solid foundation on which to build future resurrection studies.

References

Andersen, D. M., and B. A. Keafer. 1987. An endogenous annual clock in the toxic marine dinoflagellate *Gonyaulax tamarensis*. Nature **325**: 616–617. doi:10.1038/325616a0

Andersson, B., A. Godhe, H. L. Filipsson, K. Rengefors, and O. Berglund. 2020. Differences in metal tolerance among strains, populations, and species of marine diatoms-

importance of exponential growth for quantification. Aquat. Toxicol. **226**: 105551. doi:10.1016/j.aquatox.2020.105551

Andersson, B., K. Rengefors, O. Kourtchenko, K. Johannesson, O. Berglund, and H. L. Filipsson. 2022. Dataset: Cross-contamination risks in sediment-based resurrection studies of phytoplankton. doi:10.5878/9y59-7a50.

Appleby, P. G. 1998. Dating recent sediments by 210Pb: Problems and solutions, Proceeding of the 2nd NKS/EKO-1 Seminar, STUK, 7–24. https://inis.iaea.org/search/search.aspx?orig_q=RN:29040426.

Appleby, P. G. 2001. Chronostratigraphic techniques in recent sediment, p. 171–201. *In* W. M. Last and J. P. Smol [eds.], *Basin analysis, coring, and chronological techniques*. Kluwer Academic Publishers.

Beal, W. J. 1885. The viability of seeds. Proc. Soc. Promot. Agric. Sci. **5**: 44–46.

Bernhard, J. M., J. P. Barry, K. R. Buck, and V. R. Starczak. 2009. Impact of intentionally injected carbon dioxide hydrate on deep-sea benthic foraminiferal survival. Glob. Chang. Biol. **15**: 2078–2088. doi:10.1111/j.1365-2486.2008.01822.x

Burge, D. R. L., M. B. Edlund, and D. Frisch. 2018. Paleolimnology and resurrection ecology: The future of reconstructing the past. Evol. Appl. **11**: 42–59. doi:10.1111/eva.12556

Cáceres, C. E. 1998. Interspecific variation in the abundance, production, and emergence of *Daphnia* diapausing eggs. Ecology **79**: 1699–1710. doi:10.1890/0012-9658(1998)079[1699:IVITAP]2.0.CO;2

Cai, L., B. B. Jørgensen, C. A. Suttle, M. He, B. A. Cragg, N. Jiao, and R. Zhang. 2019. Active and diverse viruses persist in the deep sub-seafloor sediments over thousands of years. ISME J. **13**: 1857–1864. doi:10.1038/s41396-019-0397-9

Chen, C., and others. 2009. Seasonal changes of viable diatom resting stages in bottom sediments of Xiamen Bay, China. J. Sea Res. **61**: 125–132. doi:10.1016/j.seares.2008.11.005

Cousyn, C., L. De Meester, J. Colbourne, L. Brendonck, D. Verschuren, and F. Volckaert. 2001. Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. Proc. Natl. Acad. Sci. USA **98**: 6256–6260. doi:10.1073/pnas.111606798

Delebecq, G., S. Schmidt, A. Ehrhold, M. Latimier, and R. Siano. 2020. Revival of ancient marine dinoflagellates using molecular biostimulation. J. Phycol. **56**: 1077–1089. doi:10.1111/jpy.13010

Derry, A. M., S. E. Arnott, and P. T. Boag. 2010. Evolutionary shifts in copepod acid tolerance in an acid-recovering lake indicated by resurrected resting eggs. Evol. Ecol. **24**: 133–145. doi:10.1007/s10682-009-9295-3

Ellegaard, M., and S. Ribeiro. 2018. The long-term persistence of phytoplankton resting stages in aquatic 'seed banks'. Biol. Rev. **93**: 166–183. doi:10.1111/brv.12338

Ellegaard, M., S. Ribeiro, N. Lundholm, T. J. Andersen, T. Berge, F. Ekelund, and A. Godhe. 2013. Using the sediment archive of living dinoflagellate cysts and other protist resting stages to study temporal population dynamics, p. 149–153. *In* F. Marret, J. M. Lewis, and L. R. Bradley [eds.], *Biological and geological perspectives of dinoflagellates*. The Micropalaeontological Society.

Ellegaard, M., A. Godhe, and S. Ribeiro. 2018. Time capsules in natural sediment archives—tracking phytoplankton population genetic diversity and adaptation over multidecadal timescales in the face of environmental change. Evol. Appl. **11**: 11–16. doi:10.1111/eva.12513

Ferguson, M., and J. Ihrie. 2019. MPN: Most probable number and other microbial enumeration techniques. R package version 0.3.0. https://CRAN.R-project.org/package=MPN

Frisch, D., P. K. Morton, P. R. Chowdhury, B. W. Culver, J. K. Colbourne, L. J. Weider, and P. D. Jeyasingh. 2014. A millennial-scale chronicle of evolutionary responses to cultural eutrophication in *Daphnia*. Ecol. Lett. **17**: 360–368. doi:10.1111/ele.12237

Garrison, D. L. 1979. Monterey Bay phytoplankton I. Seasonal cycles of phytoplankton assemblages. J. Plankton Res. **1**: 241–265. doi:10.1093/plankt/1.3.241

Guillard, R. R. L. 1975. Culture of phytoplankton for feeding marine invertebrates, p. 29–60. *In* W. L. Smith and M. H. Chanley [eds.], *Culture of marine invertebrate animals*. Springer.

Hairston, N. G., Jr., R. A. Van Brunt, C. M. Kearns, and D. R. Engstrom. 1995. Age and survivorship of diapausing eggs in a sediment egg bank. Ecology **76**: 1706–1711. doi:10.2307/1940704

Härnström, K., M. Ellegaard, T. J. Andersen, and A. Godhe. 2011. Hundred years of genetic structure in a sediment revived diatom population. Proc. Natl. Acad. Sci. U. S. A. **108**: 4252–4257. doi:10.1073/pnas.1013528108

Itakura, S., I. Imai, and K. Itoh. 1997. "Seed bank" of coastal planktonic diatoms in bottom sediments of Hiroshima Bay, Seto Inland Sea, Japan. Mar. Biol **128**: 497–508. doi:10.1007/s002270050116

Jarvis, B., C. Wilrich, and P. T. Wilrich. 2010. Reconsideration of the derivation of most probable numbers, their standard deviations, confidence bounds and rarity values. J. Appl. Microbiol. **109**: 1660–1667. doi:10.1111/j.1365-2672.2010.04792.x

Karlsson, M., M. Malmaeus, E. Rydin, and P. Jonsson. 2010. Bottenundersökningar i Upplands, Stockholms, Södermanlands och Östergötlands skärgårdar 2008-2009. Rapport B1928, IVL Svenska Miljöinstitutet, Stockholm. www.ivl.se

Kerfoot, W. C., J. A. Robbins, and L. J. Weider. 1999. A new approach to historical reconstruction: Combining descriptive and experimental paleolimnology. Limnol. Oceanogr. **44**: 1232–1247. doi:10.4319/lo.1999.44.5.1232

Lennon, J. T., and S. E. Jones. 2011. Microbial seed banks: The ecological and evolutionary implications of dormancy. Nat. Rev. Microbiol. **9**: 119–130. doi:10.1038/nrmicro2504

Lewis, J., A. S. D. Harris, K. J. Jones, and R. L. Edmonds. 1999. Long-term survival of marine planktonic diatoms and dinoflagellates in stored sediment samples. J. Plankton Res. **21**: 343–354. doi:10.1080/00071618000650361

Livingstone, D., and G. Jaworski. 1980. The viability of akinetes of blue-green algae recovered from the sediments of Rostherne Mere. Brit. J. Phycol. **15**: 357–364. doi:10.1080/00071618000650361

Lundholm, N., S. Ribeiro, T. J. Andersen, T. Koch, A. Godhe, F. Ekelund, and M. Ellegaard. 2011. Buried alive–germination of up to a century-old marine protist resting stages. Phycologia **50**: 629–640. doi:10.2216/11-16.1

Lundholm, N., S. Ribeiro, A. Godhe, L. Rostgaard Nielsen, and M. Ellegaard. 2017. Exploring the impact of multidecadal environmental changes on the population genetic structure of a marine primary producer. Ecol. Evol. **7**: 3132–3142. doi:10.1002/ece3.2906

McQuoid, M. R., A. Godhe, and K. Nordberg. 2002. Viability of phytoplankton resting stages in the sediments of a coastal Swedish fjord. Eur. J. Phycol. **37**: 191–201. doi:10.1017/S0967026202003670

Montresor, M., C. Di Prisco, D. Sarno, F. Margiotta, and A. Zingone. 2013. Diversity and germination patterns of diatom resting stages at a coastal Mediterranean site. Mar. Ecol. Prog. Ser. **484**: 79–95. doi:10.3354/meps10236

Morono, Y., and others. 2020. Aerobic microbial life persists in oxic marine sediment as old as 101.5 million years. Nat. Commun. **11**: 3626. doi:10.1038/s41467-020-17330-1

Ning, W., A. Ghosh, T. Jilbert, C. P. Slomp, M. Khan, J. Nyberg, D. J. Conley, and H. L. Filipsson. 2016*a*. Evolving coastal character of a Baltic Sea inlet during the Holocene shoreline regression: Impact on coastal zone hypoxia. J. Paleolimnol. **55**: 319–338. doi:10.1007/s10933-016-9882-6

Ning, W., J. Tang, and H. L. Filipsson. 2016*b*. Long-term coastal openness variation and its impact on sediment grain-size distribution: A case study from the Baltic Sea. Earth Surf. Dyn. **4**: 773–780. doi:10.5194/esurf-4-773-2016

Ning, W., and others. 2018. Anthropogenic and climatic impacts on a coastal environment in the Baltic Sea over the last 1000 years. Anthropocene **21**: 66–79. doi:10.1016/j.ancene.2018.02.003

Pedersen, M. W., and others. 2016. Postglacial viability and colonization in North America's ice-free corridor. Nature **537**: 45–49. doi:10.1038/nature19085

Ribeiro, S., T. Berge, N. Lundholm, T. J. Andersen, F. Abrantes, and M. Ellegaard. 2011. Phytoplankton growth after a century of dormancy illuminates past resilience to catastrophic darkness. Nat. Commun. **2**: 311. doi:10.1038/ncomms1314

Sallon, S., and others. 2008. Germination, genetics, and growth of an ancient date seed. Science **320**: 1464. doi:10.1126/science.1153600

Sanyal, A., J. Larsson, F. van Wirdum, T. Andrén, M. Moros, M. Lönn, and E. Andrén. 2022. Not dead yet: Diatom resting spores can survive in nature for several millennia. Am. J. Bot. **109**: 67–82. doi:10.1002/ajb2.1780

Schmidt, S., J.-M. Jouanneau, O. Weber, P. Lecroart, O. Radakovitch, F. Gilbert, and D. Jézéquel. 2007. Sedimentary processes in the Thau lagoon (France): From seasonal to century time scales. Estuar. Coast. Shelf Sci. **72**: 534–542. doi:10.1016/j.ecss.2006.11.019

Smith, D. C., A. J. Spivack, M. R. Fisk, S. A. Haveman, H. Staudigel, and L. Party. 2000. Methods for quantifying potential microbial contamination during deep ocean coring. ODP Technical Note: No 28. doi:10.2973/odp.tn.28.2000

Stockner, J. G., and J. Lund. 1970. Live algae in postglacial lake deposits. Limnol. Oceanogr. **15**: 41–58. doi:10.4319/lo.1970.15.1.0041

Telewski, F. W., and J. A. Zeevaart. 2002. The 120-yr period for Dr. Beal's seed viability experiment. Am. J. Bot. **89**: 1285–1288. doi:10.3732/ajb.89.8.1285

Tengberg, A., and others. 1995. Benthic chamber and profiling landers in oceanography—a review of design, technical solutions and functioning. Prog. Oceanogr. **35**: 253–294. doi:10.1016/0079-6611(95)00009-6

Verardo, D. J., P. N. Froelich, and A. McIntyre. 1990. Determination of organic carbon and nitrogen in marine sediments using the Carlo Erba NA-1500 analyzer. Deep-Sea Res. Part A Oceanogr. Res. **37**: 157–165. doi:10.1016/0198-0149(90)90034-S

Acknowledgments

The Captain and the crew of R/V Electra af Askö, Raphael Gollnisch, Johan Burman, and Kotaro Hirose for sediment sampling assistance. Adele Maciute gathered the oxygen microsensor data. Rickard Stenow shared an MPN script, which served as a template for the analysis. Björn Andersson's late supervisor and mentor, Anna Godhe, who sparked my curiosity about the hidden world of phytoplankton resting stages. Anna assisted in the 2017 sampling and was the PI for this project until she passed away in 2019. She did not get the chance to see any of the work we began together in its final published form, and she did not plan, participate, or view data from the 2020 experiments. Therefore, Anna was not included as a co-author in this manuscript. The submitted manuscript benefited greatly from thoughtful comments from the editors James Cloern and Peter R. Leavitt, as well as two anonymous reviewers. This project was funded by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS) grant number 2016–00594, and the Oscar and Lili Lamm Foundation grant number FO2018-0042.

Submitted 21 December 2021
Revised 20 October 2022
Accepted 25 October 2022