

## Long-term pigment dynamics and diatom survival in dark sediment

Bart Veuger<sup>\*,1</sup> and Dick van Oevelen

Centre for Estuarine and Marine Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Yerseke, The Netherlands

### Abstract

In order to investigate survival of diatoms and long-term pigment dynamics in dark sediment, we incubated samples of homogenized, sieved, tidal-flat sediment for 1 yr in darkness. Microscopic observations revealed that some diatoms survived the full year in darkness and retained their pigments. Concentrations of the diatom-specific phospholipid-derived fatty acid (PLFA) 20:5 $\omega$ 3 indicate that these survivors represented only a small fraction of the original diatom community, probably no more than a few percent. Consequently, the effect of pigment retention in living diatoms on the overall pigment dynamics was negligible. The photosynthetic potential of the surviving diatoms was further investigated by re-exposing the sediment to light in the presence of <sup>13</sup>C-bicarbonate. Rapid <sup>13</sup>C fixation into algal PLFAs at a level similar to that in sediment freshly collected from the field indicates that surviving diatoms fully retained their photosynthetic capacity. Concentration dynamics of photosynthetic pigments generally reflected degradation of two different pools with clearly different loss rates. Losses during the first 1–2 months of the experiment (half lives of 6–22 d) reflected degradation of fresh algal material, while losses during the rest of the experiment reflected much slower degradation of more refractory pigment pools (half lives of  $\geq$  1 yr). Individual pigments showed distinctly different labilities, with losses ranging from 0% to 86% over the full year. The order of overall loss was fucoxanthin > chlorophyll *c* > chlorophyll *a* > diadinoxanthin > pheophorbide > pheophytin > diatoxanthin.

A major fraction of the organic matter that enters marine sediments is derived from algae. For sediments in shallow waters, this organic matter includes material from water-column phytoplankton as well as from benthic microalgae living at the sediment surface. Algal organic matter consists of a wide range of compounds, including photosynthetic pigments. Several of these pigments are used as biomarkers for specific algal groups and are often assumed to be specific for living algae. However, photosynthetic pigments are in general not fully degraded in sediments (Hurley and Armstrong 1990; Sun et al. 1993; Steenbergen et al. 1994), and strong differences in degradability of individual pigments can occur (Hurley and Armstrong 1990; Steenbergen et al. 1994). Like for other organic compounds, degradation of pigments is controlled by various factors (Burdige 2007). A major controlling factor is oxygen availability (Sun et al. 1993; Bianchi et al. 2000), which is often directly linked with bioturbation activity of benthic fauna (Bianchi et al. 2000). Ultimately, those pigments that escape degradation can be preserved in the sediment over geological timescales, which allows, for example, reconstruction of past phytoplankton biomass and community composition from distribution of pigments in sediment cores (Leavitt 1993; Steenbergen et al. 1994).

One aspect of bio(geo)chemical studies on the dynamics of photosynthetic pigments in sediments that often appears to be overlooked is retention of pigments in *living* algae, especially in the absence of light. Algae, in particular diatoms, have been shown to be able to survive in dark sediment over long periods of time (months to years)

(Smayda and Mitchell-Innes 1974; Itakura et al. 1997; Lewis et al. 1999), and viable diatoms have been reported to be present in deep waters well below the photic zone (Malone et al. 1973; Cahoon et al. 1994; McGee et al. 2008) and deep in the sediment (Steele and Baird 1968; Cahoon et al. 1994). Mechanisms for survival of algae during prolonged periods of darkness include reduction of metabolic activity (Jochem 1999), formation of resting stages (Durbin 1978; Sugie and Kuma 2008), and facultative heterotrophy (Lewin 1953; Tuchman et al. 2006). During dark survival, diatoms appear to retain their pigments (Peters 1996; Murphy and Cowles 1997). This means that pigments in living diatoms can contribute to the total pigment pool in sediments, including those in deep, dark waters. Hansen and Josefson (2003) investigated the contribution of living planktonic diatoms from a water-column spring bloom to the total sediment chlorophyll *a* concentrations and found that chlorophyll *a* was actually predominantly present in living diatoms.

The retention of pigments by diatoms during long periods of darkness suggests that diatoms also retain their photosynthetic capacity. Various studies have found that diatoms can resume their activity when re-exposed to light after long periods in the dark (Smayda and Mitchell-Innes 1974; Itakura et al. 1997), and photosynthetic activity upon re-exposure to light after long periods of darkness has been measured for pelagic diatom cultures (Peters 1996; Murphy and Cowles 1997). However, the retention of the photosynthetic capacity of natural communities of benthic microalgae (BMA) has never been investigated. Natural BMA communities may be particularly well adapted for survival in the dark given their regular exposure to periods of darkness as a result of the dynamic conditions on tidal-flat sediments (e.g., submersion with turbid water, sedimentation, bioturbation).

\* Corresponding author: b.veuger@nioo.knaw.nl

<sup>1</sup> Present address: Analytical and Environmental Chemistry, Vrije Universiteit Brussel, Brussel, Belgium

In the present study, we investigated survival of diatoms, retention of their photosynthetic capacity, and dynamics of photosynthetic pigments. A 1-yr incubation experiment was performed with tidal-flat sediment samples in darkness. In order to specifically investigate survival and degradation processes involved without bias from physical disturbances typically encountered in the field (faunal activity, tidal currents, etc.), sediment was sieved to remove macrofauna and incubated *in vitro*. Diatom survival and pigment dynamics were assessed by analysis of concentrations of photosynthetic pigments and phospholipid-derived fatty acids (PLFAs) during the 1-yr incubations. The retention of the photosynthetic capacity of surviving diatoms was investigated in an additional experiment in which sediment was re-exposed to light in the presence of  $^{13}\text{C}$ -bicarbonate, and  $^{13}\text{C}$  incorporation into diatom PLFAs was measured to quantify photosynthetic activity.

## Methods

Results presented in this manuscript are part of a 1-yr experiment that was set up to investigate the long-term fate of microbial C and N in sediment. In this study, the natural microbial community of a tidal-flat sediment was labeled with  $^{13}\text{C}$  and  $^{15}\text{N}$  (by exposure to  $^{13}\text{C}$ -glucose and  $^{15}\text{N}$ -ammonium), after which the fate of the  $^{13}\text{C}$  and  $^{15}\text{N}$  in and concentrations of various compounds was followed over time. Stable-isotope tracer and concentration data will be presented elsewhere (B. Veuger unpubl.), while the current manuscript specifically deals with concentrations of photosynthetic pigments. In addition, this paper also includes some PLFA concentration data to aid interpretation of the dynamics of pigments, algal biomass, and total microbial biomass.

In order to further investigate the potential photosynthetic activity of diatoms after 1 yr in darkness, a dedicated experiment was performed in which sediment that had spent a full year in darkness was re-exposed to light in the presence of  $^{13}\text{C}$ -dissolved inorganic carbon (DIC). The  $^{13}\text{C}$  fixation into diatom PLFAs was used as a measure of photosynthetic activity.

*Sediment collection*—Sediment was collected from a tidal mud flat (Paulinapolder) in the midregion of the turbid, nutrient-rich, and heterotrophic Scheldt Estuary (the Netherlands). The sampling site is characteristic for tidal flats in this part of the estuary. The BMA community is typically dominated by diatoms, and an annual BMA bloom occurs on these tidal flats in spring (April to May), and a smaller bloom happens in early autumn (September) (Barranguet et al. 1997). Further characteristics of the sediment and full details on the experimental setup can be found elsewhere (B. Veuger unpubl.). Briefly, on 14 June 2006, 20 small sediment cores (2.5 cm inner diameter, 10 cm deep) were collected from the tidal flat at low tide. Sediment from the cores was pooled and transported to the laboratory.

*Incubations*—Freshly collected sediment was suspended in water from the sampling site and sieved (1 mm mesh) to

remove most of the macrofauna. Sieved sediment was allowed to settle overnight in the dark. The following morning, excess water was removed, the remaining sediment slurry was homogenized, and 40-mL subsamples were transferred to 100-mL glass bottles. Another 20 mL of field water and 0.38 mL of a solution containing 21 mmol  $\text{L}^{-1}$   $^{13}\text{C}$ -glucose and 27 mmol  $\text{L}^{-1}$   $^{15}\text{N}$ -ammonium were added, and bottles were shaken. These amounts represent trace levels, meaning that these additions did not substantially change total concentrations of dissolved organic carbon (DOC) and ammonium in the sediment. After addition of the labeled substrates, 20-mL aliquots of water from the sampling site were added to each bottle, and the bottles were shaken and closed with open-top screw caps with a silicone liner (allowing gas exchange through the caps without loss of water). The sediment quickly settled to form a 25–30-mm-thick layer with a similar layer of water on top. Labeled sediments were incubated in the dark at 17°C for 1, 4, 7, 18, 30, 69, 133, 250, and 371 d. Two bottles were sacrificed for sampling at each sampling time. Sampling involved shaking the bottles, taking a subsample for PLFA analysis (directly into extraction solvent), and freezing the rest of the slurry (–35°C). Frozen sediment was later freeze-dried and stored frozen (–35°C) until extraction of pigments. On d 371, additional subsamples were fixed with formol and examined by fluorescence microscopy on the same day. In order to test whether –35°C ensured proper preservation of pigments, identical sets of sediment samples were stored at –35°C and –80°C for several months and later analyzed for pigment concentrations.

*$^{13}\text{C}$ -DIC incubations*—In order to investigate the photosynthetic potential of surviving diatoms after long-term exposure to darkness, an additional set of unlabeled sediment samples (treated the same as the labeled samples, except for addition of the labeled substrates) was incubated together with the labeled sediments. After 1 yr of incubation in the dark, these two bottles with unlabeled sediment (40 mL of sediment + 40 mL of water) were shaken, and resulting slurries were transferred to glass culture flasks (20 cm diameter), which resulted in a thin layer of sediment (~ 1 mm) with a similar layer of water on top.

In order to compare results for the “old” sediment that had been incubated in darkness for 1 yr, we simultaneously performed identical incubations with “fresh” sediment that was collected from the same field location as the samples collected the year before. This sediment was sampled, converted to a slurry, and transferred to culture flasks following the same procedure as the year before (i.e., sediment from 0–10-cm cores pooled, 2 bottles filled with 40 mL of sediment + 40 mL of freshly sampled water from field site per bottle). Fresh sediment was not sieved. However, there was no visible presence or activity of macrofauna in the culture flasks.

Both the fresh and old sediment were placed under artificial light (23  $\mu\text{Einstein m}^{-2} \text{s}^{-1}$ ) at 18°C. After ~ 20 h of pre-incubation in the light, 5-mL aliquots of a solution containing  $^{13}\text{C}$ -labeled sodium bicarbonate (0.37 mmol  $\text{L}^{-1}$ ,

about 10% of ambient DIC concentrations) were added to all four flasks. After gentle shaking, sediments were incubated in the light for another 2.5 h before sampling. Sampling involved shaking the flasks, followed by collection of subsamples of the resulting sediment slurry for  $^{13}\text{C}$ -PLFA analysis (6 mL of slurry transferred directly into extraction solvent). The remaining material was frozen ( $-35^\circ\text{C}$ ), freeze-dried (4 d later), and subsequently stored at  $-35^\circ\text{C}$  until pigment extraction. In addition, some fresh sediment from the field site was directly sampled (i.e., not incubated with  $^{13}\text{C}$ -DIC) and used as control sample to determine the natural abundance of  $^{13}\text{C}$  in the PLFAs.

**Pigment extraction and analysis**—Pigments were extracted from freeze-dried sediment with 90% acetone in Milli-Q water with intense shaking. Extracts were analyzed by high-performance liquid chromatography (HPLC) (Novapak C18 column) using fluorescence and photodiode array (PDA) detection. Pigments were identified by comparison of retention times and PDA absorption spectra with those of standards. Concentrations could be determined in all samples for the following pigments: chlorophyll *a*, chlorophyll *c*, diadinoxanthin, diatoxanthin, fucoxanthin, pheophytin, and pheophorbide. Concentrations of chlorophyll *a*, pheophytin, and pheophorbide include various allomers of these respective pigments.

**PLFA extraction and analysis**—PLFAs were extracted from  $\sim 6$ -mL samples of sediment slurry in chloroform-methanol-water using a modified Bligh and Dyer method (Boschker 2004). The total lipid extract was fractionated on silica gel into different classes by polarity. The most polar fraction, containing the PLFAs, was derivatized by mild methanolysis yielding fatty acid methyl esters (FAMES), which were analyzed by gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) to determine concentrations and  $^{13}\text{C}$ -enrichment ( $\delta^{13}\text{C}$ ) (see Boschker [2004] for details). For samples from the long-term experiment, PLFAs were extracted directly after sampling and from freeze-dried samples. Analysis of PLFA concentrations in directly extracted samples from d 133 and 250 was unsuccessful.

**PLFA data treatment**—PLFA data for the long-term experiment primarily serve as additional data to aid interpretation of the pigment dynamics and algal survival. For the long-term incubations, concentrations of the diatom biomarker 20:5 $\omega$ 3 for the directly extracted samples were used despite missing data for d 133 and 250 because comparison of PLFA concentrations in directly sampled vs. freeze-dried sediment revealed substantial loss (60–90%) of the algal PLFA 20:5 $\omega$ 3 in freeze-dried samples (much more than for other PLFAs). Concentrations of total and summed bacterial PLFAs from freeze-dried sediment were used as proxies for microbial biomass dynamics over time.

For the short-term  $^{13}\text{C}$ -DIC incubations, we present all PLFAs that could be identified and properly quantified in both the old and fresh sediment. The  $^{13}\text{C}$ -labeling data are presented as  $\Delta\delta^{13}\text{C}$  (relative  $^{13}\text{C}$ -enrichment) and excess  $^{13}\text{C}$  (absolute amount of  $^{13}\text{C}$ ). The  $\Delta\delta^{13}\text{C}$  value was

calculated as:  $\Delta\delta^{13}\text{C} = (\delta^{13}\text{C} \text{ in labeled sample}) - (\delta^{13}\text{C} \text{ in control sample})$ . The  $\delta^{13}\text{C}$  value was calculated as:  $\delta^{13}\text{C} = (\text{R}_{\text{sample}}/\text{R}_{\text{standard}} - 1) \times 1000$ , where  $\text{R} = ^{13}\text{C}:^{12}\text{C}$  and  $\text{R}_{\text{standard}} = 0.011180$ . Excess  $^{13}\text{C}$  per gram dry sediment was calculated as:  $\text{excess } ^{13}\text{C} = ([\text{at}\%^{13}\text{C}_{\text{sample}} - \text{at}\%^{13}\text{C}_{\text{control}}]/100) \times (\text{concentration C in sample})$ .  $\text{At}\%^{13}\text{C}$  was calculated from  $\delta^{13}\text{C}$  as:  $\text{at}\%^{13}\text{C} = (100 \times \text{R}_{\text{standard}} \times [\delta^{13}\text{C}_{\text{sample}}/1000] + 1)/(1 + \text{R}_{\text{standard}} \times [\delta^{13}\text{C}_{\text{sample}}/1000] + 1)$ .

**Data fitting**—Concentrations of the individual pigments and PLFA 20:5 $\omega$ 3 over time ( $C_t$ ) were fitted with a double-exponential decay model:  $C_t = C_0(fe^{-k_f t} + [1 - f]e^{-k_s t})$ , in which the pigment concentration at time zero ( $C_0$ ) is assumed to consist of a fast-decaying ( $f$ ) and a slow-decaying ( $1 - f$ ) fraction, which both decrease exponentially over time with respective degradation rates  $k_f$  and  $k_s$ . This function was used to fit the individual raw data points in R (R Development Core Team 2010) using the function *nl*, which returns the optimal parameter values and their statistical significance level. Half lives were calculated as  $(\ln 2)/k$ .

## Results

**Microscopic observations**—Examination of sediment samples by fluorescence microscopy revealed the presence of fluorescent diatoms in the sediment from the long-term dark incubations. Cell densities were not quantified.

**Pigment concentrations**—Various pigments were detected in the fresh sediment in the following order of abundance: pheophorbide > chlorophyll *a* > pheophytin > fucoxanthin > diadinoxanthin  $\approx$  diatoxanthin  $\approx$  chlorophyll *c* (see Fig. 1 for concentrations). The relative composition of this pigment pool is consistent with a BMA community dominated by diatoms, as previously reported for sediments in the midregion of the Scheldt Estuary (Barranguet et al. 1997).

Concentrations of the individual pigments in the long-term dark incubations showed different trends over time. However, all were well described by the double-exponential degradation model, except for diatoxanthin (Fig. 1). Fucoxanthin, chlorophyll *c*, chlorophyll *a*, diadinoxanthin, pheophorbide, and pheophytin showed a net decrease over time, with a rapid exponential decrease during the first weeks and a much slower decrease, or even complete leveling off (in the case of diadinoxanthin) during the rest of the experiment, while concentrations for diatoxanthin remained more or less steady over time. The size of the fast-decaying pigment fraction varied greatly among the pigments, ranging between 0.17 and 0.74 ( $f$  values in Fig. 1), and were ordered as fucoxanthin > diadinoxanthin  $\approx$  chlorophyll *c*  $\approx$  chlorophyll *a* > pheophorbide  $\approx$  pheophytin. The order of loss-rate constants for this fast-decaying pigment pool ( $k_f$  values in Fig. 1) was chlorophyll *c*  $\gg$  pheophytin > pheophorbide > fucoxanthin = chlorophyll *a* > diadinoxanthin. These values correspond to half lives of 6 d for chlorophyll *c* to 22 d for diadinoxanthin. The order of loss-rate constants for the slow-degrading fractions ( $k_s$  values in Fig. 1) was fucoxanthin = chlorophyll *c* > chlorophyll *a*  $\gg$  pheophytin  $\approx$

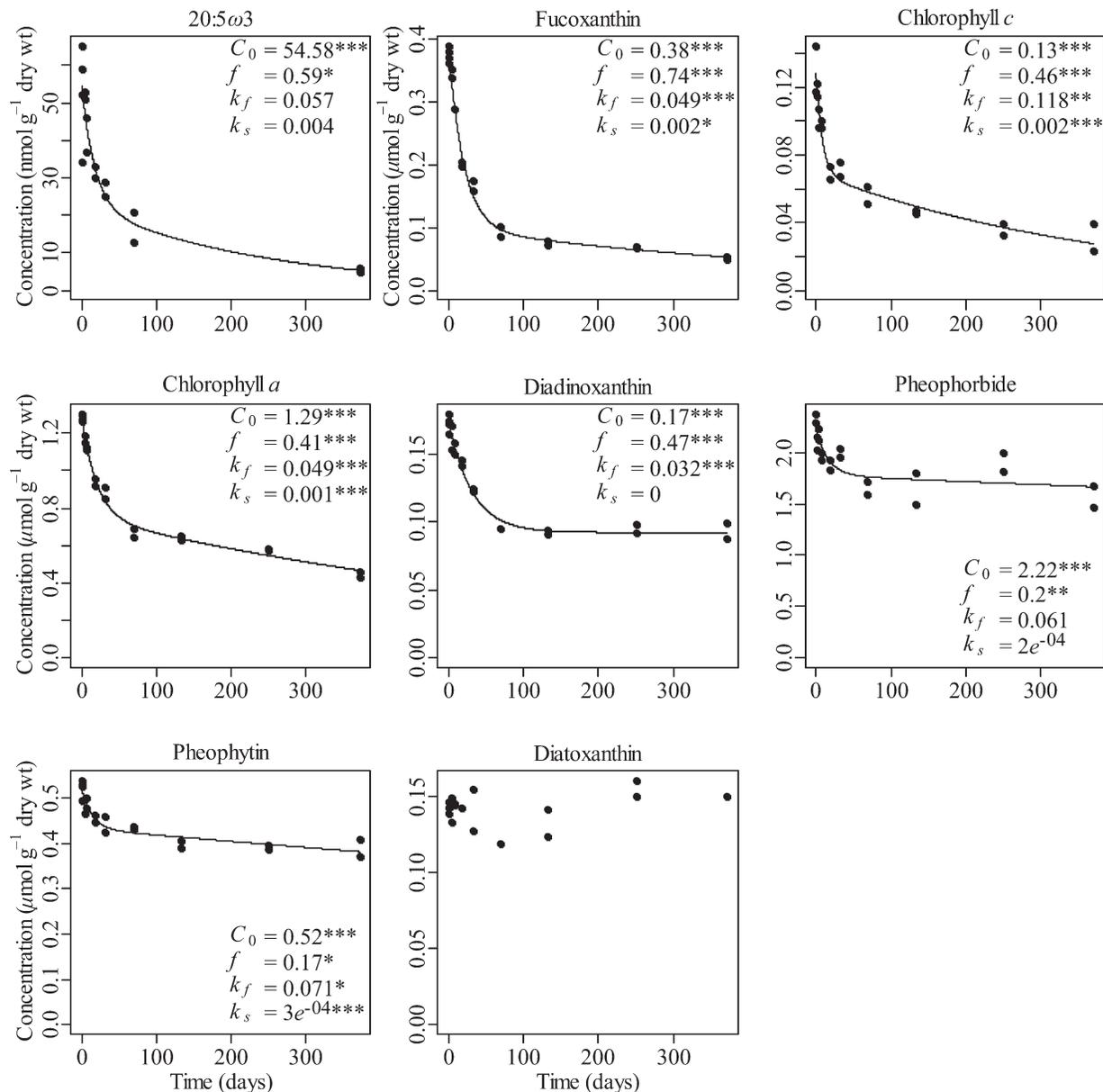


Fig. 1. Concentrations of pigments and diatom PLFA 20:5 $\omega$ 3 during 1-yr incubation in darkness fitted with double-exponential decay model. Parameters from fits are indicated in panels.  $C_0$  = total concentration at d 0,  $f$  = fast-degrading fraction of  $C_0$ ,  $k_f$  = loss-rate constant of fast-degrading fraction,  $k_s$  = loss-rate constant of slow-degrading fraction ( $1 - f$ ), dry wt = dry weight. Asterisks indicate corresponding significance levels (\*\*\*)  $0 < p < 0.001$ , (\*\*)  $0.001 < p < 0.01$ , (\*)  $0.01 < p < 0.05$ ).

pheophorbide. These rates correspond to half lives of 347 d for fucoxanthin and chlorophyll *c* to 3466 d for pheophorbide. The order in overall loss over the full experiment was: fucoxanthin (86% loss) > chlorophyll *c* (76%) > chlorophyll *a* (66%) > diadinoxanthin (46%) > pheophorbide (33%) > pheophytin (26%).

The ratio between degradation products pheophytin and pheophorbide vs. chlorophyll *a* gradually increased from 2.0 to 4.4 during the experiment (Fig. 2). The ratio between diatoxanthin vs. diadinoxanthin showed a similar increase from 0.8 to 1.6, with values leveling off toward the end of the experiment (Fig. 2).

Comparison of concentrations of a selection of pigments in identical samples that had been stored at  $-35^\circ\text{C}$  vs.

$-80^\circ\text{C}$  generally showed the same concentrations (chlorophyll *a* and diatoxanthin), or even slightly higher concentrations (chlorophyll *c*, pheophytin, and fucoxanthin) for samples that had been stored at  $-35^\circ\text{C}$ . Recoveries ([concentration in  $-35^\circ\text{C}$  sample/concentration in  $-80^\circ\text{C}$  sample]  $\times$  100) were all between 98% and 113%. These results are consistent with those by Reuss and Conley (2005) and confirm that the results for our primary set of samples (that had been stored at  $-35^\circ\text{C}$ ) were not biased by pigment loss during sample storage.

**PLFA concentrations**—Diatom PLFA 20:5 $\omega$ 3 showed a rapid exponential decrease during the first weeks and a much slower decrease during the rest of the experiment

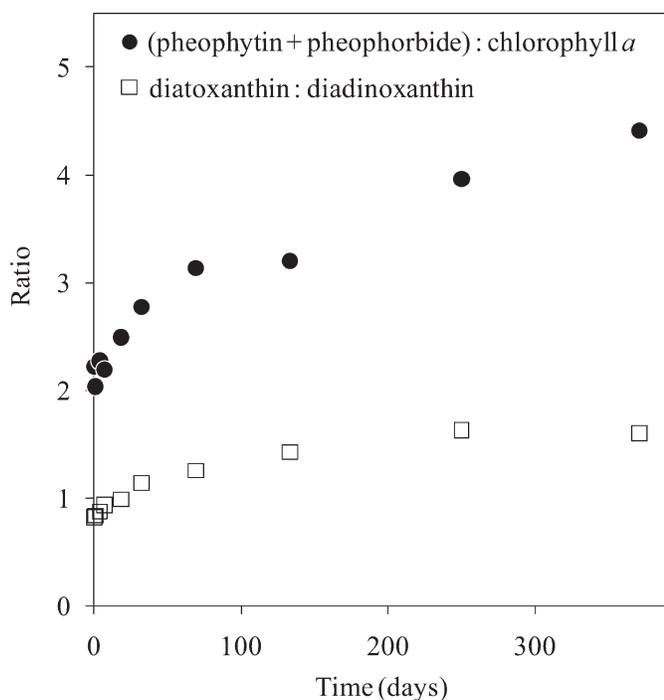


Fig. 2. Ratios of pheophytin + pheophorbide vs. chlorophyll *a* (closed circles) and diatoxanthin vs. diadinoxanthin (open squares) over time.

(Fig. 1). The size of the rapidly lost fraction ( $f$ ) and its loss-rate constant ( $k_f$ ) were comparable to those for fucoxanthin, chlorophyll *a*, and chlorophyll *c*, while the loss-rate constant for the slow-degrading fraction ( $k_s$ ) was 2–4 times higher than that of these pigments (Fig. 1). The overall loss of 20:5 $\omega$ 3 over the whole experiment was 90%, which is very similar to that of fucoxanthin (86%).

Concentrations of total PLFAs and summed bacterial PLFAs remained at a rather stable level throughout the experiment (Fig. 3), especially when compared with the dynamics of 20:5 $\omega$ 3 and the pigments in Fig. 1. Total PLFAs include all PLFAs in Fig. 4, except for 18:2 $\omega$ 6 $c$ , and with the addition of *i*14:0, *i*15:0, and *ai*15:0. Summed bacterial PLFAs comprise *i*14:0, *i*15:0, *ai*15:0, and 18:1 $\omega$ 7 $c$  (see Boschker and Middelburg 2002). Concentrations decreased somewhat during the first weeks, followed by an increase up to values somewhat higher than those at the start of the experiment at d 133 and steady values thereafter (Fig. 3).

**<sup>13</sup>C-DIC incubations after 1 yr**—Addition of <sup>13</sup>C-DIC to the sediment in the culture flasks incubated in the light resulted in measurable <sup>13</sup>C-fixation in PLFAs. The  $\Delta\delta^{13}\text{C}$ -PLFA profiles were similar for old and fresh sediment, with  $\Delta\delta^{13}\text{C}$  values of up to 6–36‰ for the old sediment and 2–45‰ for the fresh sediment (Fig. 4a). PLFAs 16:2 $\omega$ 4, 16:3 $\omega$ 4, 20:4 $\omega$ 6, and 20:5 $\omega$ 3, which are considered specific for diatoms (Dijkman and Kromkamp 2006), showed substantially higher  $\Delta\delta^{13}\text{C}$  values for the old sediment than for the fresh sediment (Fig. 4a). The <sup>13</sup>C-enrichment of bacteria-specific PLFAs *i*14:0, *i*15:0, and *ai*15:0 was below the limit of detection. The  $\Delta\delta^{13}\text{C}$ -PLFA profiles were similar for old and fresh sediment (Fig. 4a). PLFA

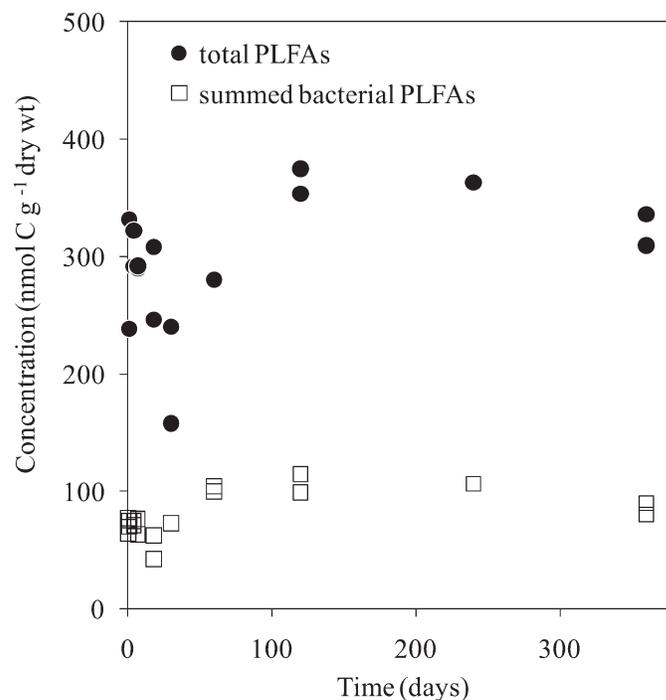


Fig. 3. Concentrations of total PLFAs (closed circles) and summed bacterial PLFAs (open squares) over time.

concentrations were on average 4 times higher in the fresh sediment. Excess <sup>13</sup>C (the absolute amount of <sup>13</sup>C) in the summed PLFAs was 4.4 times higher in the fresh sediment, and excess <sup>13</sup>C labeling profiles (Fig. 4b) were similar for fresh and old sediment, with 16:0, 16:1 $\omega$ 7 $c$ , and 18:1 $\omega$ 7 $c$  being the dominant PLFAs. Pigment concentrations did not show a measurable change during the 22.5-h light incubations.

## Discussion

Survival of diatoms during long periods of darkness and degradation of photosynthetic pigments in sediments are two topics that have so far been studied in isolation. In the present study, we investigated both these topics, and potential concomitant effects, in a single experiment. We also investigated the retention of the photosynthetic capacity of the surviving diatoms, a topic that has so far remained unstudied. The three topics will be discussed in separate sections next.

**Diatom survival**—Survival of diatoms in the sediment during the full year in the dark was directly evident from the observed presence of living, fluorescent diatoms in the sediment at the end of the experiment. However, analysis of concentrations of the diatom-specific PLFA 20:5 $\omega$ 3 revealed that only a very small fraction of the original diatom community survived.

The observed survival of diatoms is consistent with results from previous studies in which diatoms have been shown to be able to survive in dark, cold, sediment for periods of up to several years (Smayda and Mitchell-Innes 1974; Itakura et al. 1997; Lewis et al. 1999). Studies on

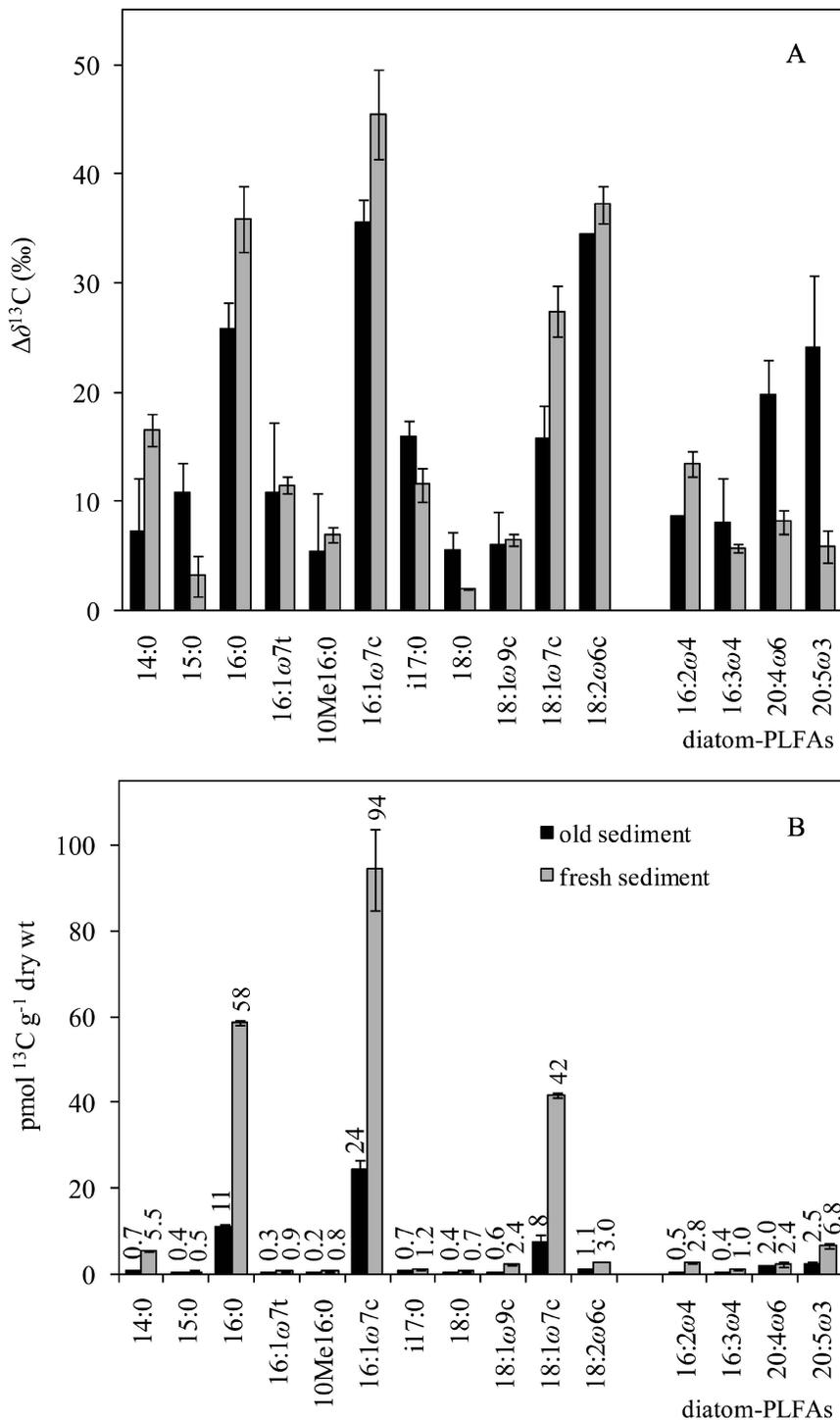


Fig. 4. (A)  $\Delta\delta^{13}\text{C}$  values and (B) excess  $^{13}\text{C}$  for PLFAs in short-term incubations with  $^{13}\text{C}$ -bicarbonate in the light for “old” sediment that had been incubated in the dark for 1 yr (black bars) and “fresh” sediment directly from the field (gray bars). Diatom PLFAs are grouped on right-hand side. (B) Values on bars are precise values. Error bars are standard deviations.

diatom survival in sediments often focus on the formation of resting stages, including resting cells and resting spores or cysts (cells specifically adapted for survival). Formation of resting stages and subsequent burial in the sediment represent a well-studied survival strategy of pelagic diatoms

during periods of unfavorable conditions (low temperature, nutrient limitation, etc.). In this way, the sediment serves as a “seed bank” for pelagic diatoms (Itakura et al. 1997). Alternatively, diatoms can also survive prolonged periods of darkness as viable (active) cells by reducing their cellular

metabolism and/or by heterotrophy. Heterotrophy appears to be a rather common feature, because many diatom species can grow on organic substrates in the dark (Lewin 1953; Tuchman et al. 2006). In the present study, heterotrophy may well have been a more important survival mechanism than the formation of resting stages, given the rapid response in the light incubations (indicating that diatoms were present as vegetative cells rather than resting stages). Moreover, the incubation temperature was relatively high (17°C) and similar to in situ summer temperatures, while survival in the form of resting stages is generally associated with low temperatures, and resting-stage survival decreases with increasing temperature (Smayda and Mitchell-Innes 1974; Peters 1996).

Further quantification of the fraction of the original diatom community that survived during the 1-yr incubation requires a good proxy for living diatom biomass. Even though pigments are regularly used as biomarkers for phytoplankton, their incomplete degradation in sediments (see following discussion) complicates their use as a proxy for living *benthic* algae. Therefore, the best available proxy for living diatoms in the present study is the concentration of PLFA 20:5 $\omega$ 3 (Fig. 1), which is the dominant PLFA in diatoms (Dijkman and Kromkamp 2006). PLFAs are comparatively rapidly degraded upon cell death and are therefore often considered to be specific for living biomass (Boschker and Middelburg 2002). However, membrane lipids generally degrade much slower under anoxic conditions than under oxic conditions (Harvey et al. 1986; Ding and Sun 2005). Even though the water overlying the sediment remained oxygenated, oxygen penetration into the organic-rich, muddy, sediment from which the majority of the macrofauna had been removed was probably limited to the upper few millimeters. Consequently, degradation must have occurred predominantly under anoxic conditions (except for the upper few millimeters), which means that 20:5 $\omega$ 3 was likely not rapidly degraded upon cell death and hence that estimates of living diatom biomass based on concentrations of 20:5 $\omega$ 3 are probably overestimates. Therefore, the following estimates of diatom survival should be considered as maximum values.

The rapid decrease in concentrations of 20:5 $\omega$ 3 during the first 1–2 months (Fig. 1) indicates that a large fraction of the living diatoms that were present in the freshly collected sediment died quickly after the start of the incubation and were degraded. The 70% decrease over the first 3 months and 90% decrease over the full year indicate that maximum diatom survival was 30% and 10% after 3 months and 1 yr, respectively. Taking into account the incomplete degradation of 20:5 $\omega$ 3 under anoxic conditions, this suggests that only a small fraction, probably no more than a few percent, of the original diatom community survived the full year in darkness.

*Potential photosynthetic capacity after 1 yr*—The presence of *fluorescent* diatoms in the sediment that had spent a full year in the dark indicated that surviving diatoms had retained their pigments and hence also retained their photosynthetic capacity. This was confirmed by results from the short-term  $^{13}\text{C}$ -DIC incubations in the light, in which

photosynthetic activity of the surviving diatoms was actually similar to that of diatoms freshly collected from the field.

The measured  $^{13}\text{C}$  incorporation in the PLFAs (Fig. 4) demonstrates that  $^{13}\text{C}$  had been incorporated in microbial biomass, which may have included diatoms as well as bacteria. The higher absolute  $^{13}\text{C}$  incorporation (excess  $^{13}\text{C}$ ; Fig. 4b) in the fresh sediment primarily reflects the higher biomass (PLFA concentrations) in the fresh sediment. Out of the various PLFA profiles for different algal groups, the  $^{13}\text{C}$ -labeled PLFA profile (Fig. 4b) best matches the profile of diatoms (see Dijkman and Kromkamp 2006). This is consistent with diatoms being the dominant algal group in the studied sediment. However, the most abundant PLFA in diatoms is 20:5 $\omega$ 3 (Dijkman and Kromkamp 2006), while the  $^{13}\text{C}$ -labeled profile was actually dominated by 16:0, 16:1 $\omega$ 7c, and 18:1 $\omega$ 7c. This was most likely the result of additional  $^{13}\text{C}$  fixation by bacteria. The taxonomic resolution of the  $^{13}\text{C}$ -PLFA profile is not sufficient to determine the exact identity of the bacteria involved. However, this bacterial  $^{13}\text{C}$  incorporation most likely involved direct  $^{13}\text{C}$ -DIC fixation by chemoautotrophic bacteria. The  $^{13}\text{C}$  incorporation by heterotrophic bacteria (indirectly via uptake of  $^{13}\text{C}$ -DOC released by labeled algae) appears to have been negligible, since there was no measurable  $^{13}\text{C}$  incorporation into PLFAs specific for heterotrophic bacteria (*i*14:0, *i*15:0, and *ai*15:0).

The best measure for cell-specific  $^{13}\text{C}$ -fixation activity of the diatoms is provided by the  $\Delta\delta^{13}\text{C}$  values for the diatom markers (Fig. 4a). The  $\Delta\delta^{13}\text{C}$  values for the diatom markers in the old sediment were similar to (16:2 $\omega$ 4, 16:3 $\omega$ 4), or even substantially higher than (20:4 $\omega$ 6 and 20:5 $\omega$ 3), those in the fresh sediment (Fig. 4a). This indicates that the cell-specific photosynthetic activity of the surviving diatoms was at the same level as that of those from the freshly sampled sediment. These results suggest that surviving diatoms completely retained their photosynthetic capacity during a full year in darkness.

To the best of our knowledge, the present study is the first to report on the retention of photosynthetic capacity of a natural benthic diatom community. For pelagic algae, a few studies have investigated the effect of long periods of darkness on their photosynthetic capacity, and results are variable. Laboratory experiments with pelagic diatom cultures have revealed a strong reduction in the photosynthetic carbon assimilation potential during a 35-d incubation period in darkness for algae from the temperate North Sea (Peters 1996). Murphy and Cowles (1997) investigated the photosynthetic capacity of a pelagic diatom by directly measuring in vivo multi-excitation chlorophyll *a* fluorescence of a *Thalassiosira weissflogii* culture during 2 months of darkness and found a strong retention of photosynthetic capacity. Tang et al. (2009) investigated the retention of photosynthetic capacity of *Phaeocystis antarctica* under dark and cold conditions and found a substantial reduction of photosynthetic capacity over several months, and also that recovery of photosynthetic capacity required 20 d upon re-exposure to light. Altogether, results from these studies on pelagic algae confirm the ability of algae to retain their photosynthetic capacity up to several months in darkness. Compared to these results for pelagic algae, the

benthic diatoms in the present study responded very rapidly (within hours) when re-exposed to light, and retention of photosynthetic capacity was very high (capacity similar to that of diatoms in freshly sampled sediment). These results indicate that benthic diatoms are very capable of dealing with exposure to longer periods of darkness. Together with heterotrophy, this is one of the features that enables benthic diatoms to live in tidal-flat sediments that regularly experience periods of darkness, both for short periods (hours) due to, for example, submersion with turbid water, and for longer periods (days to months) due to burial by, for example, sedimentation and bioturbation.

*Pigment dynamics*—Earlier herein, we inferred from the dynamics of PLFA 20:5 $\omega$ 3 that only a small fraction of the initial diatom community survived the full year of darkness. Consequently, pigments retained in living cells likely comprised only a very small fraction of the total pigment pool, meaning that their effect on the overall pigment dynamics was negligible.

Similar to the dynamics of 20:5 $\omega$ 3, the dynamics of pigment concentrations are generally well fitted with a double-exponential degradation model (Fig. 1). These dynamics, as well as corresponding loss rates, are similar to those reported for degradation of chlorophyll *a* in sediments by Sun et al. (1991, 1993). The double-exponential decrease indicates that total pigment pools consisted of two subpools with clearly different degradabilities. The rapidly degrading pool (half lives of 6–22 d) likely consisted of a fresh pool of relatively labile pigments derived from algae that died during the first days of the experiment. The slowly decaying pool (half lives of  $\geq 1$  yr) was a more refractory pool that likely included a refractory pigment pool from the algae that died at the start of the experiment as well as a relatively refractory background pool that was already present in the sediment in a nonliving form upon sampling from the field.

The measured pigment dynamics may have been influenced by changes in microbial activity and/or redox conditions during the experiment. However, concentrations of total and summed bacterial PLFAs (Fig. 3) indicate that microbial biomass remained relatively constant throughout the experiment, especially when compared to algal biomass (20:5 $\omega$ 3 in Fig. 1). Combined with typical bacterial turnover times of 1–100% d<sup>-1</sup> for surface sediments (Sander and Kalff 1993), this suggests that microbial activity also remained at a steady level. The latter factor combined with the oxygen saturation of the overlying water and the absence of faunal activity point to steady redox conditions during the experiment. Altogether, it seems that the effect of changes in microbial activity and redox conditions on the pigment dynamics was minor.

Even though our data do not allow full assessment of all pigment subpools and degradation mechanisms, net results do show a clear order of degradability for individual pigments. The general order of degradability in the present study is consistent with results from previous studies on pigment degradation in natural phytoplankton assemblages (Leavitt and Carpenter 1990), as well as those inferred from compositional differences in pigment pools of sinking

organic matter in the water column vs. that in the sediment (Hurley and Armstrong 1990; Steenbergen et al. 1994), those inferred from sediment depth profiles (Stephens et al. 1997), and those inferred from a general comparison of pigments pools in algae (Jeffrey et al. 1997) vs. sediments (Barranguet et al. 1997; Woulds and Cowie 2009).

Out of the analyzed suite of pigments, fucoxanthin displayed the most labile character and best correlated with 20:5 $\omega$ 3, indicating that fucoxanthin is the most suitable biomarker pigment for diatoms in sediments. The labile character of fucoxanthin may be related to the presence of epoxide functional groups (Repeta and Gagosian 1987). Chlorophyll *a*, chlorophyll *c*, and diadinoxanthin showed rather similar trends, except for the complete leveling off of diadinoxanthin after 2 months (Fig. 1). The latter indicates that the less-degradable component of the sediment diatoxanthin pool was very refractory.

The dynamics of pheophytin and pheophorbide are consistent with these two pigments being chlorophyll degradation products. The rapid losses from the fresh material during the first weeks confirm that pheophytin and pheophorbide were also rapidly degraded themselves and hence were only intermediates in chlorophyll degradation. In contrast, after this rapid degradation, the remaining refractory background pool was relatively large and degraded slowly compared to most of the intact pigments. Clearly, different mechanisms were involved in the degradation of the fresh material and that of the background pool. The relatively low ratio between pheophytin + pheophorbide and chlorophyll *a* (2.0–4.4; Fig. 2) compared to, for example, values of 12–27 reported for deep-sea sediments (Woulds and Cowie 2009) indicates that the observed pigment dynamics reflect an early stage of diagenesis.

Diatoxanthin had the most refractory nature, with no net loss during the experiment (Fig. 1). This highly refractory character may be partly explained by production of diatoxanthin as a degradation product of diadinoxanthin (Louda et al. 2002), similar to the aforementioned production of pheophytin and pheophorbide from chlorophylls. The absence of an increase in diatoxanthin concentrations indicates that diatoxanthin itself was also degraded and hence was only an intermediate step in the degradation of diadinoxanthin.

#### *Acknowledgments*

We thank Pieter van Rijswijk, Marco Houtekamer, Peter van Breugel, Cobie van Zetten, and Anton Tramper for their help with field and laboratory work.

B.V. was financially supported by the Netherlands Organization for Scientific Research (Pionier 833.02.2002) and the Darwin Center for Biogeology (project 142.16.1052). D.v.O. was supported by the Hotspot Ecosystem Research and Man's Impact on European Seas (HERMIONE) project (grant agreement 226354) funded by the European Community's Seventh Framework Programme (FP7/2007–2013).

This is Netherlands Institute of Ecology (NIOO-KNAW) publication 4981.

#### *References*

- BARRANGUET, C., P. M. J. HERMAN, AND J. J. SINKE. 1997. Microphytobenthos biomass and community composition studied by pigment biomarkers: Importance and fate in the

- carbon cycle of a tidal flat. *J. Sea Res.* **38**: 59–70, doi:10.1016/S1385-1101(97)00032-4
- BIANCHI, T. S., B. JOHANSSON, AND R. ELMGREN. 2000. Breakdown of phytoplankton pigments in Baltic sediments: Effects of anoxia and loss of deposit-feeding macrofauna. *J. Exp. Mar. Biol. Ecol.* **251**: 161–183, doi:10.1016/S0022-0981(00)00212-4
- BOSCHKER, H. T. S. 2004. Linking microbial community structure and functioning: Stable isotope ( $^{13}\text{C}$ ) labeling in combination with PLFA analysis, p. 1673–1688. *In* G. A. Kowalchuk, F. J. de Bruijn, I. M. Head, A. D. Akkermans, and J. D. van Elsas [eds.], *Molecular microbial ecology manual II*. Kluwer.
- , AND J. J. MIDDELBURG. 2002. Stable isotopes and biomarkers in microbial ecology. *FEMS Microbiol. Ecol.* **40**: 85–95, doi:10.1111/j.1574-6941.2002.tb00940.x
- BURDIGE, D. J. 2007. Preservation of organic matter in marine sediments: Controls, mechanisms, and an imbalance in sediment organic carbon budgets? *Chem. Rev. (Washington, DC)* **107**: 467–485. doi:10.1002/chin.200720266
- CAHOON, L. B., R. A. LAWS, AND C. J. THOMAS. 1994. Viable diatoms and chlorophyll *a* in continental slope sediments off Cape Hatteras, North Carolina. *Deep-Sea Res. II* **41**: 767–782, doi:10.1016/0967-0645(94)90047-7
- DIJKMAN, N. A., AND J. C. KROMKAMP. 2006. Phospholipid-derived fatty acids as chemotaxonomic markers for phytoplankton: Application for inferring phytoplankton composition. *Mar. Ecol. Prog. Ser.* **324**: 113–125, doi:10.3354/meps324113
- DING, H., AND M. Y. SUN. 2005. Biochemical degradation of algal fatty acids in oxic and anoxic sediment–seawater interface systems: Effects of structural association and relative roles of aerobic and anaerobic bacteria. *Mar. Chem.* **93**: 1–19, doi:10.1016/j.marchem.2004.04.004
- DURBIN, E. G. 1978. Aspects of the biology of resting spores of *Thalassiosira nordenskiöldii* and *Detonula confervacea*. *Mar. Biol.* **45**: 31–37, doi:10.1007/BF00388975
- HANSEN, J. L. S., AND A. B. JOSEFSON. 2003. Accumulation of algal pigments and live planktonic diatoms in aphotic sediments during the spring bloom in the transition zone of the North and Baltic Seas. *Mar. Ecol. Prog. Ser.* **248**: 41–54, doi:10.3354/meps248041
- HARVEY, H. R., R. D. FALLON, AND J. S. PATTON. 1986. The effect of organic matter and oxygen on the degradation of bacterial membrane lipids in marine sediments. *Geochim. Cosmochim. Acta* **50**: 795–804, doi:10.1016/0016-7037(86)90355-8
- HURLEY, J. P., AND D. E. ARMSTRONG. 1990. Fluxes and transformations of aquatic pigments in Lake Mendota, Wisconsin. *Limnol. Oceanogr.* **35**: 384–398, doi:10.4319/lo.1990.35.2.0384
- 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
- JEFFREY, S. W., R. F. C. MANTOURA, AND S. W. WRIGHT [EDS.]. 1997. *Phytoplankton pigments in oceanography: Guidelines to modern methods*. Monographs on oceanographic methodology, v. 10. UNESCO Publishing.
- JOCHEM, F. J. 1999. Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate. *Mar. Biol.* **135**: 721–728, doi:10.1007/s002270050673
- LEAVITT, P. R. 1993. A review of factors that regulate carotenoid and chlorophyll deposition and fossil pigment abundance. *J. Paleolimnol.* **9**: 109–127, doi:10.1007/BF00677513
- , AND S. R. CARPENTER. 1990. Aphotic pigment degradation in the hypolimnion: Implications for sedimentation studies and paleolimnology. *Limnol. Oceanogr.* **35**: 520–534, doi:10.4319/lo.1990.35.2.0520
- LEWIN, J. C. 1953. Heterotrophy in diatoms. *J. Gen. Microbiol.* **9**: 305–313. doi:10.1099/00221287-9-2-305
- LEWIS, J., A. HARRIS, K. JONES, AND R. EDMONDS. 1999. Long-term survival of marine planktonic diatoms and dinoflagellates in stored sediment samples. *J. Plankton Res.* **21**: 343–354, doi:10.1093/plankt/21.2.343
- LOUDA, J. W., L. LIU, AND E. W. BAKER. 2002. Senescence- and death-related alteration of chlorophylls and carotenoids in marine phytoplankton. *Org. Geochem.* **33**: 1635–1653, doi:10.1016/S0146-6380(02)00106-7
- MALONE, T. C., C. GARSIDE, R. ANDERSON, AND O. A. ROELS. 1973. The possible occurrence of photosynthetic microorganisms in deep-sea sediments of the North Atlantic. *J. Phycol.* **9**: 482–488.
- MCGEE, D., R. A. LAWS, AND L. B. CAHOON. 2008. Live benthic diatoms from the upper continental slope: Extending the limits of marine primary production. *Mar. Ecol. Prog. Ser.* **356**: 103–112, doi:10.3354/meps07280
- MURPHY, A. M., AND T. J. COWLES. 1997. Effects of darkness on multi-excitation in vivo fluorescence and survival in a marine diatom. *Limnol. Oceanogr.* **42**: 1444–1453, doi:10.4319/lo.1997.42.6.1444
- PETERS, E. 1996. Prolonged darkness and diatom mortality: II. Marine temperate species. *J. Exp. Mar. Biol. Ecol.* **207**: 43–58, doi:10.1016/0022-0981(95)02519-7
- R DEVELOPMENT CORE TEAM. 2010. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org>.
- REPETA, D. J., AND R. B. GAGOSIAN. 1987. Carotenoid diagenesis in recent marine sediments: I. The Peru continental shelf (15°S, 75°W). *Geochim. Cosmochim. Acta.* **51**: 1001–1009, doi:10.1016/0016-7037(87)90111-6
- REUSS, N., AND D. J. CONLEY. 2005. Effects of sediment storage conditions on pigment analyses. *Limnol. Oceanogr.: Methods* **3**: 477–487, doi:10.4319/lom.2005.3.477
- SANDER, B. C., AND J. KALFF. 1993. Factors controlling bacterial production in marine and freshwater sediments. *Microb. Ecol.* **26**: 79–99, doi:10.1007/BF00177045
- SMAYDA, T. J., AND B. MITCHELL-INNES. 1974. Dark survival of autotrophic, planktonic marine diatoms. *Mar. Biol.* **25**: 195–202, doi:10.1007/BF00394965
- STEELE, J. H., AND I. E. BAIRD. 1968. Production ecology of a sandy beach. *Limnol. Oceanogr.* **13**: 14–25, doi:10.4319/lo.1968.13.1.0014
- STEENBERGEN, C. L. M., H. J. KORTHALS, AND E. G. DOBRYNIN. 1994. Algal and bacterial pigments in non-laminated lacustrine sediment: Studies of their sedimentation, degradation and stratigraphy. *FEMS Microbiol. Ecol.* **13**: 335–351, doi:10.1111/j.1574-6941.1994.tb00080.x
- STEPHENS, M. P., D. C. KADKO, C. R. SMITH, AND M. LATASA. 1997. Chlorophyll-*a* and pheopigments as tracers of labile organic carbon at the central equatorial Pacific seafloor. *Geochim. Cosmochim. Acta.* **61**: 4605–4619, doi:10.1016/S0016-7037(97)00358-X
- SUGIE, K., AND K. KUMA. 2008. Resting spore formation in the marine diatom *Thalassiosira nordenskiöldii* under iron- and nitrogen-limited conditions. *J. Plankton Res.* **30**: 1245–1255, doi:10.1093/plankt/fbn080
- SUN, M. Y., R. C. ALLER, AND C. LEE. 1991. Early diagenesis of chlorophyll-*a* in Long Island Sound sediments: A measure of carbon flux and particle reworking. *J. Mar. Res.* **49**: 379–401, doi:10.1357/002224091784995927
- , C. LEE, AND R. C. ALLER. 1993. Laboratory studies of oxic and anoxic degradation of chlorophyll-*a* in Long Island

- Sound sediments. *Geochim. Cosmochim. Acta.* **57**: 147–157, doi:[10.1016/0016-7037\(93\)90475-C](https://doi.org/10.1016/0016-7037(93)90475-C)
- TANG, K. W., W. O. SMITH, JR., A. R. SHIELDS, AND D. T. ELLIOTT. 2009. Survival and recovery of *Phaeocystis antarctica* (Prymnesiophyceae) from prolonged darkness and freezing. *Proc. Biol. Sci.* **276**: 81–90, doi:[10.1098/rspb.2008.0598](https://doi.org/10.1098/rspb.2008.0598)
- TUCHMAN, N. C., M. A. SCHOLLETT, S. T. RIER, AND P. GEDDES. 2006. Differential heterotrophic utilization of organic compounds by diatoms and bacteria under light and dark conditions. *Hydrobiologia* **561**: 167–177, doi:[10.1007/s10750-005-1612-4](https://doi.org/10.1007/s10750-005-1612-4)
- WOULDS, C., AND G. L. COWIE. 2009. Sedimentary pigments on the Pakistan margin: Controlling factors and organic matter dynamics. *Deep-Sea Res. II* **56**: 347–357, doi:[10.1016/j.dsr2.2008.05.033](https://doi.org/10.1016/j.dsr2.2008.05.033)

*Associate editor: Robert E. Hecky*

*Received: 26 July 2010*

*Accepted: 26 January 2011*

*Amended: 16 February 2011*