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Vertical migration behaviour of diatom assemblages of Wadden Sea sediments (Dangast, Germany): a study using cryo-scanning electron microscopy

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Summary The vertical migration behaviour of diatom assemblages inhabiting Wadden Sea sediments near Dangast (Germany) was investigated using cryo-scanning electron microscopy. The diatom assemblages were dominated by small *Navicula* species. Intertidal sediments which were located at different distances from the high tide level or stayed submerged even throughout low tides were chosen. Samples were prepared and cryofixed in the field. Sampling was restricted to three sets: (i) before the onset of vertical migration, (ii) 3 to 5 h after the onset of vertical migration, and (iii) before the area became flooded again or just prior to dusk. The diatom assemblages inhabiting the different types of sediments did not always show the same response. When the tidal cycle exposed the sediment surfaces during the night cell densities increased in the early morning hours with the onset of light. Later on, although the photon flux density was still increasing, cell densities stayed constant or decreased before the water flooded the areas around noon. In experiments in which the water drained off around noon and the areas became exposed throughout the entire afternoon, cell densities increased even up to dusk when the photon flux density had dropped to values below 20 µM photons m^{-2} s^{-1}. In an experiment in which the last sampling occured at 10.15 pm, when the photon flux density had already declined below 10 µM photons m^{-2} s^{-1}, cell densities had decreased to lower values. This was ca. 1 h before the area was flooded again. Finally, cryo-scanning electron microscopy revealed frequently occuring micropatches of diatom assemblages which could be differentiated into typical areas of lower and higher cell densities further complicating the pattern of light or water cover induced movements.

Key words Diatom assemblages · Vertical migration · Cryo-scanning electron microscopy · Wadden Sea sediments · Navicules

Introduction

The phenomenon of vertical migration (VM) is known since the beginning of this century [4], and circumscribes the rhythmic migration of benthic microorganisms onto the sediment surface when the water drains off and back with incoming tide. The dimensions in which VM occurs are generally low and do not exceed 2 mm in muddy sediments [12]. VM has been described for several phototrophic microorganisms, such as cyanobacteria, diatoms, dinophytes and euglenophytes [2, 5, 8, 10, 11, 18, 19]. Light, tidal rhythm, geotaxis and probably other, yet undefined external and/or internal variables seem to induce and/or influence this migration behaviour [cf. 18]. In most of the experiments approaching this phenomenon cell enumeration was conducted by direct cell counting, counting of live cells by epi-(auto)fluorescence or by the lens tissue technique (LTT) [1–3]. The LTT documents almost exclusively cells active in movement, whereas cryo-scanning electron microscopy (Cryo-SEM), which has proven to be an excellent technique for in situ studies of natural assemblages, does not differentiate between moving and immotile cells and thus reveals a static picture of all cells present on the sediment surface. Cryo-SEM has already been applied to Wadden Sea sediments inhabited by cyanobacteria, diatoms or euglenoid

algae [16] and to cyanobacteria dominated epilithic biofilms on tropical rocky shores [9] and has allowed to differentiate and classify some organisms even down to the species level. Furthermore, it was demonstrated that measurements of cell densities (i.e. cell counting) by Cryo-SEM studies were similar to data sets obtained by the LTT [13]. Cryo-SEM, finally, has already been used for a time scheduled study on the VM of a Wadden Sea inhabiting diatom assemblage [12]. However, the latter and most of the other experiments on VM had been performed on sediment cores or laboratory tidal micro-ecosystems.

As no experiments on VM were conducted directly with samples prepared during fieldtrips, we applied Cryo-SEM on diatom assemblages inhabiting Wadden Sea sediments near Dangast (Germany). The experiments were conducted when low tides occurred either at night or in the afternoon, thus exposing the sediment surfaces at different daytimes and to different light regimes.

Materials and methods

Field site and sampling conditions Field samples were withdrawn from muddy intertidal sediments (IS) located on the North Sea shore near the harbour of Dangast (Germany). During the study diatoms were almost the only phototrophic algal group inhabiting the muddy sediments. The dominant diatoms were small *Navicula* species. This was ascertained by light microscopy and Cryo-SEM of sediment samples. Samples were taken in May and June 1997. The sampling dates and some physical variables are compiled in Table 1. Patches, at least 15 to 20 cm in diameter and homogeneously brownish, were chosen.

Table 1 Dates and onsets of sunrise, sunset and high-tides of the three sampling days

	May 7 1997	May 20 1997	June 18 1997
Sunrise	5.37 am	5.15 am	4.53 am
Sunset	9.07 pm	9.29 pm	10.00 pm
High tides	2.05 am/2.34 pm	0.16 am/12.46 pm	11.29 am/12.04 pm
Photon flux* (max/min)	1/730	21/550	4.5/2100

*Photon flux is expressed in µM photons m^{-2} s^{-1}.

Cryo-SEM preparation Sampling was performed by cutting sediment samples in squares of approximately 8 x 8 mm and transfering them onto U-shaped self-made copper support foils (1 mm thick). The samples were 4 to 8 mm thick. The dark zones below were almost completely devoid of diatoms. This was confirmed by Cryo-SEM on freeze fractured sediment samples and by light microscopical examinations of slides of parahistological thin sectioned and resin-embedded sediment cores (data not shown). Cryofixation was achieved by plunging the copper foils into liquid propane (–184°C), and then cooling them by liquid N_2 (–196°C). Afterwards the samples were stored in dewars filled with liquid N_2. They were transferred to the laboratory and kept in liquid N_2 until Cryo-SEM examination.

For examining the VM of diatom assemblages during low-tide, samples from the same local patches were removed in regular intervals and prepared in the same way. Three samples were taken in parallel, each patch at each time. Furthermore three types of intertidal sediments (IS) were compared: (i) IS-1: intertidal sediments with diatom assemblages that stayed submerged even during low-tide (water filled holes, cavities, foot steps in the muddy sediments). These assemblages exhibited a dark brown colour throughout the entire time of observation. (ii) IS-2: intertidal sediments adjacent to IS-1 with diatom assemblages which were completely air-exposed when the water drained off and became reflooded with incoming tide. (iii) IS-3: sediments close to the high water border line. They became submerged like those of IS-2 but only during a short period of time due to their location near the high-tide level.

Three samples were taken in parallel each time and the three types of IS were investigated simultaneously. The procedure was limited to only three sampling days: on May 7 the first sampling was performed immediately before sunrise (before photon flux exceeded 10 µM photons m^{-2} s^{-1} [or: µEinstein m^{-2} s^{-1}]), then approximately 2 to 3 h later and finally before tidal immersion. On May 20 and June 18 the samples were taken immediately after the water had drained off, approx. 3 h later and finally when photon fluxes had reached values below 20 or 10 µM photons m^{-2} s^{-1}, respectively.

Cryo-SEM All manipulations with the cryofixed samples were performed either under liquid nitrogen, in a vacuum or in a dry argon atmosphere at temperatures below –95°C. The copper foils with the samples were mounted onto specimen holders and transferred into an Oxford CT1500C cryochamber (Oxford, Wiesbaden, Germany) attached to a Hitachi S3200N scanning electron microscope. Samples were etched on the cryostage at –95°C to remove surface water. After etching, the samples were Sputter-coated with approximately 10–20 nm Au and finally examined at –180°C in the scanning electron microscope operating at 20 kV.

The diatoms were counted on photographic prints of pictures taken at magnifications of 150–500x . The prints were enlarged to final magnifications of 300–1000x . As three samples were taken each time, a minimum of 10 pictures photographed from each sampled surface could be used for cell density counting. A transparent foil with a 4 x 4 cm counting area was positioned arbitrarily onto the prints thus allowing to calculate cell densities per cm^2.

Light measurements Photon flux rates were measured with a Li-Cor LI-185B Quantum/Radiometer/Photometer equipped with a LI-192SB Underwater Quantum Sensor. Measured values are given in µM photons m^{-2} s^{-1}.

Results

General observations Cryo-preparations were conducted using propane as freezing agent. The procedure of cryofixation was not time-consuming and could be easily done in the field. Cryo-SEM evidenced the general conservation of the sediment structure and the preservation of the diatom cells (see Fig. 1C). Although sediment areas which appeared to be homogeneously brownish were chosen, this turned out to be not the case. Thus, Cryo-SEM revealed that the intertidal diatom patches exhibited areas which often differed significantly in cell densities (up to 150-fold). This cannot be judged on the field site without microscopic control. A photograph showing the two areas simultaneously is given in Fig. 1B. This phenomenon was more pronounced on May 7 and less on the two other sampling dates. These areas most probably represent local "micropatches" of diatom assemblages. We therefore differentiated the patches sampled in those of higher cell density (hcd-areas) and lower cell density (lcd-areas) and calculated the cell densities for lcd- and hcd-areas of each sampling site during the study separately.

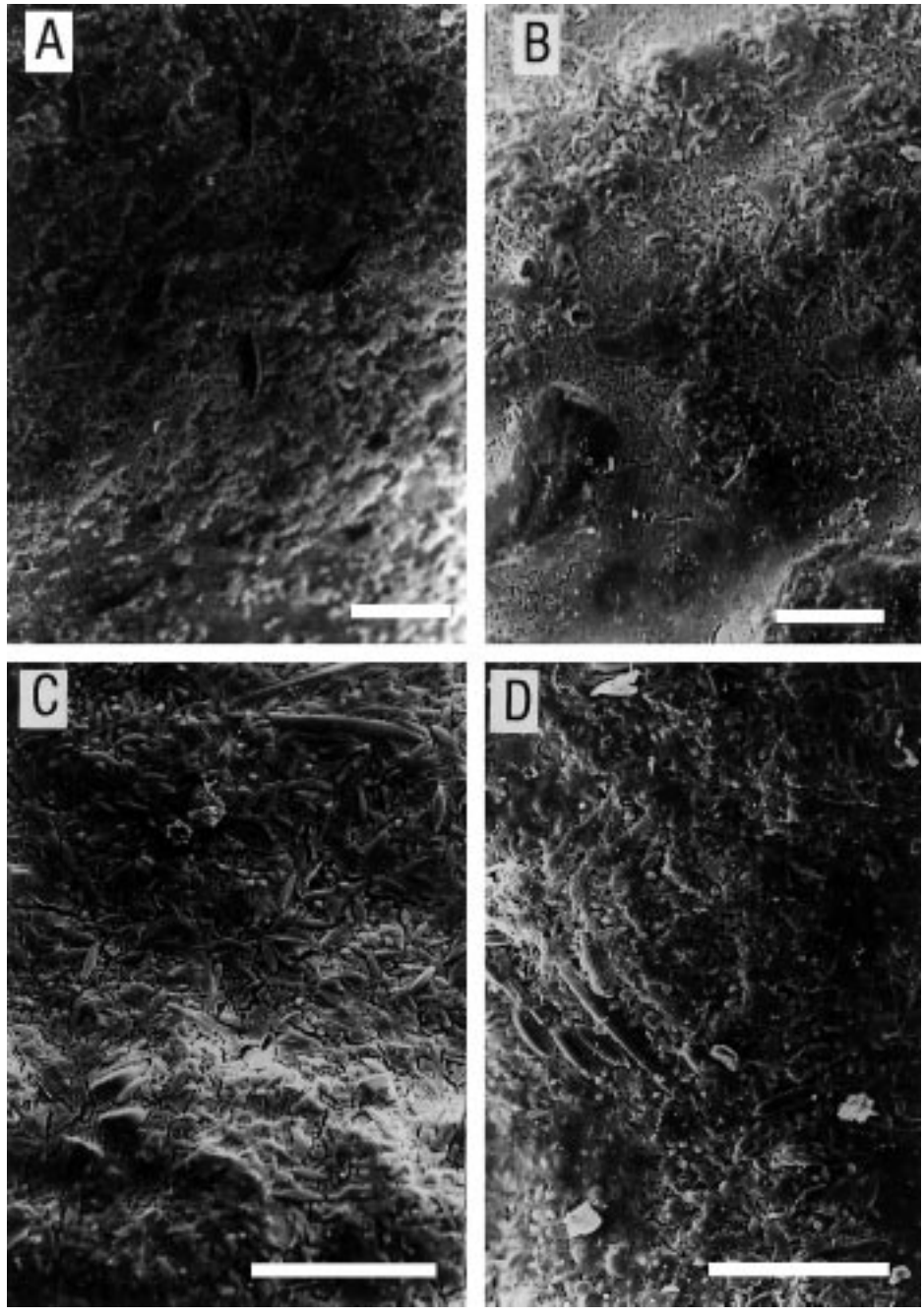

Fig. 1 Photographs obtained from Cryo-SEM examinations. Figures A to D are representative pictures of samples from the same sampling site (IS-2) which were withdrawn on June 18 at 2.00 pm (A), 5.15 pm (B), 8.15 pm (C), and 10.00 pm (D), respectively. Vertical Migration had occurred. The differences in cell densities are obvious. In B the different areas of low cell density (lcd) and high cell density (hcd) can be seen simultaneously. Bar = 100 µm

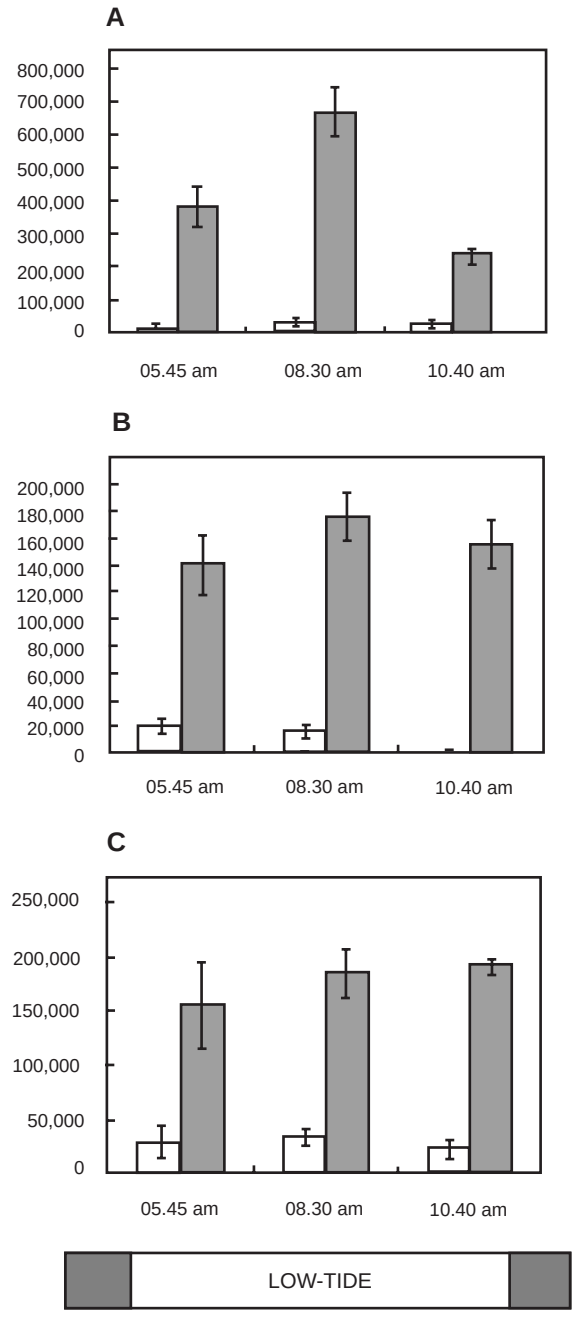

Fig. 2 Results of the Cryo-SEM examination on May 7 1997. Different types of intertidal sediments were sampled. IS-1: intertidal sediments adjacent to IS-2 with diatom assemblages that stayed submerged even during low tide; IS-2: intertidal sediments with diatom assemblages which were completely air-exposed when the water drained off and became reflooded with incoming tide; IS-3: sediments which became submerged only during a short period of time due to their location near the high water border line. IS-2 sediments were not as close to the high water border line as IS-3. Cell densities are expressed as cells/cm^2 and given for IS-1 (A), IS-2 (B) and IS-3 (C) and herein for areas of low cell densities (lcd-areas, white bars) and high cell densities (hcd-areas, stripped bars) separately. The tides are indicated below

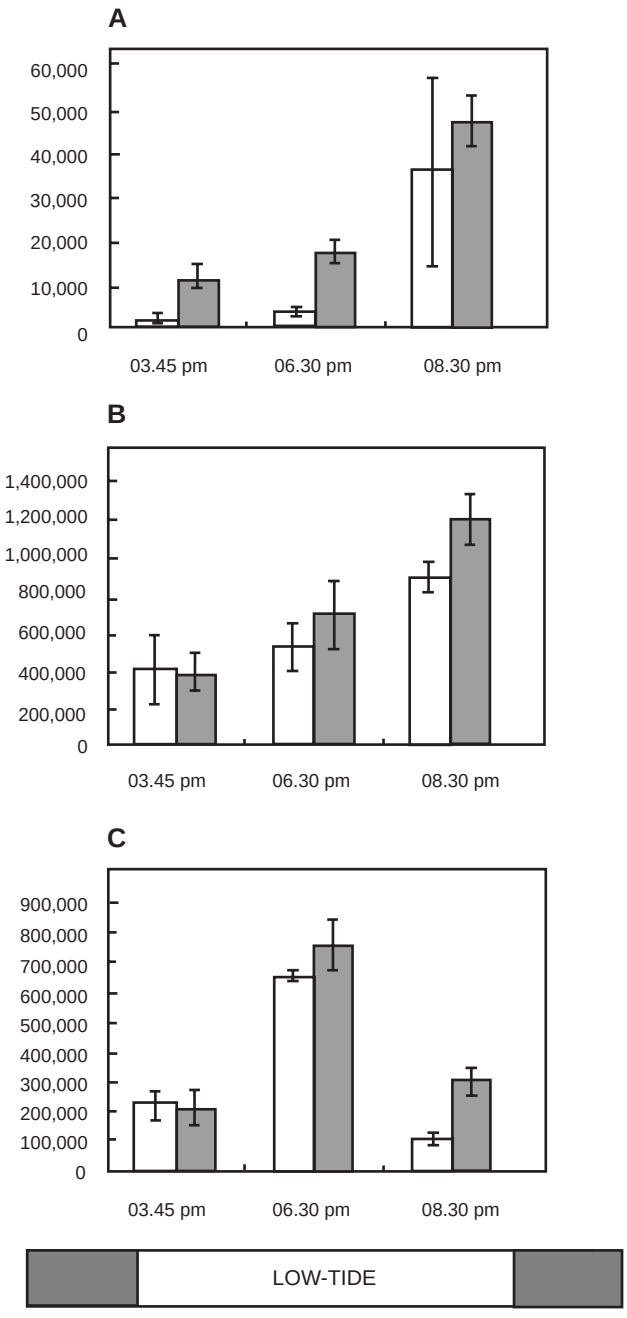

Fig. 3 Results obtained by Cryo-SEM on May 20 1997. Cell densities are expressed as cells/cm^2. For details for A, B and C see Fig. 2

However, VM was obvious from Cryo-SEM investigation as the cell densities varied significantly (see Figs. 2, 3 and 4). Representative photographs of an IS2-area sampled on June 18, 1997 at 02.00 pm, 05.15 pm, 08.15 pm and 10.00 pm are shown in Fig. 1A, B, C and D, respectively.

Cryo-SEM investigations on May 7, 1997 On this day high tide occurred in the night so that the water had already drained off from the sediments prior to sunrise (Table 1). The mud flats became flooded around noon. The photon flux rates increased from 5.30 pm continuously up to 730 µM photons m^{-2} s^{-1} measured at 10.30 am. Minor variations were caused by clouds passing by (data not shown).

The results of our Cryo-SEM investigations are compiled in Fig. 3. The overall cell densities ranged from 1000 to 34,000 cells per cm^2 in lcd-areas and from 140,000 to 660,000 cells per cm^2 in hcd-areas. In the sediments which stayed submerged even at low tide (IS-1) cell densities increased approx. 2-fold from 6.45 am to 8.30 am and decreased to values of approx. 20,000 (lcd) and 240,000 (hcd) cells per cm^2 at 10.40 am. This was found for both, lcd- and hcd- areas of IS-1, they being more pronounced in the hcd-area (Fig. 2A). IS-2, which became exposed to air during low-tide, exhibited the same result for the hcd-areas, whereas we registered a continuous decrease in cell density for its lcd-areas (Fig. 2B). In the sampling area which became covered by water only during a short period of time due to its location near the high water border line (IS-3) cell densities increased slightly throughout the entire morning in the hcd-area whereas the lcd-areas showed similar results as obtained for IS-1 (Fig. 2C).

Cryo-SEM investigations on May 20, 1997 On that day water drained off around noon thus exposing the sediment surfaces immediately to photon flux densities of approx. 400 to 600 µM photons m^{-2} s^{-1}. The curve of the photon flux density showed some minor variations due to cloudiness within the first three hours (data not shown). Photon flux density dropped from values of 500 µM photons m^{-2} s^{-1} around 6.00 pm to values below 20 µM photons m^{-2} s^{-1} at 8.30 pm. The cell densities measured by Cryo-SEM and compiled in Fig. 3 increased in IS-1 and IS-2 in hcd- and lcd-areas until the last sampling at 8.30 pm (Fig. 3A and 3B). The values calculated for IS-1 increased from approx. 2,000 cells per cm^2 to approx. 50,000 cells per cm^2. The cell densities calculated for IS-2 were much higher than those for IS-1 and increased 3-fold, from approx. 400,000 cells per cm^2 to ca. 1,000,000–1,200,000 cells per cm^2. For IS-3 we registered an approx. 4-fold increase in cell density between the samples taken at 3.45 pm and 6.30 pm. The values in the last sample taken at 8.30 pm dropped down to those of the first sampling. (Fig. 3C). At 8.30 pm the photon flux density had already decreased to values of 20 µM photons m^{-2} s^{-1}. Both, IS-3 and IS-2 could hardly be differentiated into lcd- and hcd-areas on that day.

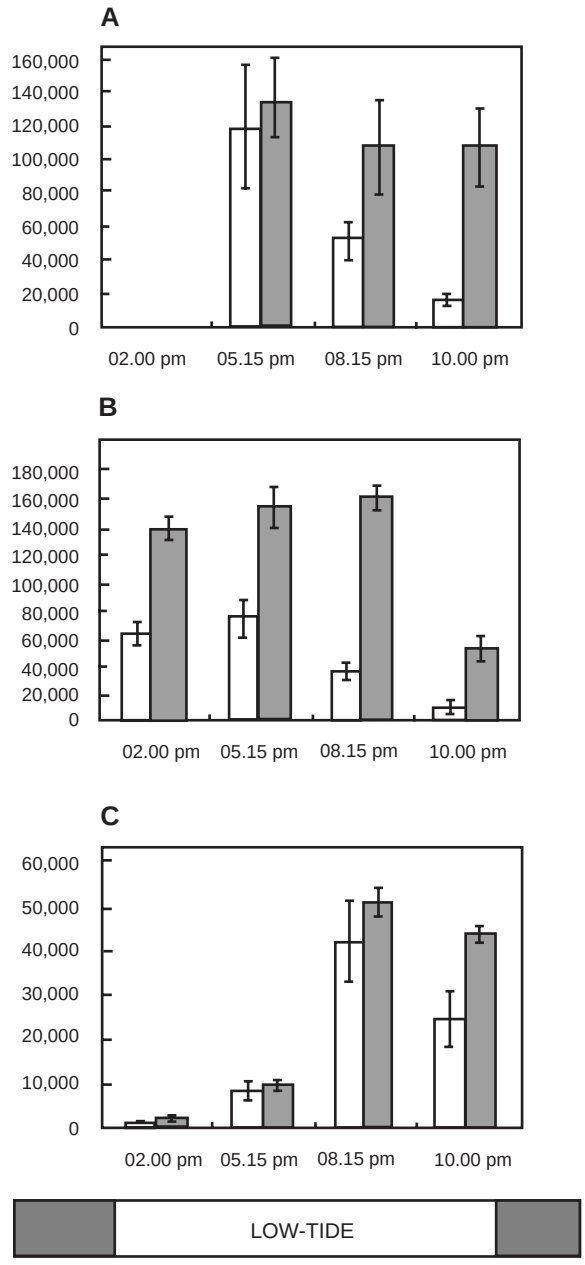

Fig. 4 Results of the Cryo-SEM examination on June 18 1997. Cell densities are expressed as cells/cm^2. For details for A, B and C see Fig. 2

Cryo-SEM investigations on June 18, 1997 Like on May 20, water drained off around noon so that the sediment surfaces became immediately exposed to light. The photon flux densities were much higher on this day and decreased from approx. 2000 µM photons m^{-2} s^{-1} measured at 2.30 pm to values below 10 µM photons m^{-2} s^{-1} at 10.00 pm (data not shown). We observed the VM without using any optical instrument as the sediment surfaces turned from a pale brown to a greyish dark brown. This

change in colour occurred within 1 h. Our measurements confirmed this observation in part. The cell densities measured by Cryo-SEM are compiled in Fig. 4. Cell densities in IS-2 and IS-3 increased until 8.15 pm but dropped in the 10.00 pm samples (Fig. 4B and 4C). The increase was more pronounced in IS-3 (approx. 40-fold), whereas the decrease at 10.00 pm was more dramatic for IS-2 (approx. 3-fold). Sampling at 2.00 pm for IS-1 was missed for technical reasons. IS-1 deviated from the two others sampling sites in that it showed a faint decrease in cell density on its hcd-areas and a more pronounced reduction in its lcd-areas (Fig. 4C). The latter results matched those found for the lcd-area of IS-3.

Discussion

Cryo-SEM has shown to be an excellent technique for in situ studies of biofilms and complex communities such as the diatom assemblages of the Wadden Sea sediments. Sediment samples are quickly cryofixed, stored in liquid N_2 and finally, transferred to the cryostage of a scanning electron microscope where free water is sublimated from the sediment. After Au-sputtering the surfaces, fractured planes can be examined at low temperatures [9, 12–14]. To achieve good preservation of structures by cryofixation, the samples have to be plunged into melting N_2 cooled to approx. –200°C. The N_2 slush is usually obtained after the liquid N_2 has been frozen in a vacuum chamber. Thus, major prerequisites for the cryo-preparation in N_2 slush are a slush chamber and a vacuum pump. This might cause problems during field trips and outdoor sampling. As a consequence, propane was used as freezing agent for cryo-preparations in the field. Cryo-fixation with propane is unexpensive, not time-consuming and can be easily performed in the field. The only devices needed are a propane flask, a dewar filled with liquid N_2 and a metal stick with a small cavity on one end functioning as a reservoir for liquefied propane. Examinations in the Cryo-SEM evidenced that both, sediment structure and cells, had been well preserved. In the course of this study, a new device, the cryolander, was constructed that improves cryo-fixation and reduces artifacts which can occur during the freezing process, thus allowing fine scale in situ sampling of surface sediments [20].

The spatial microheterogeneity in cell densities, even within a small area, was surprising. This finding is interpreted as local micropatches of diatom assemblages. The micropatches with higher cell densities might deviate from those of lower cell densities in sediment structure and/or local higher nutrient concentrations thus offering better adhesion and/or growth conditions to diatom cells. Similar findings are evident in micrographs of [21]. Micropatches probably cause high standard deviations when cell densities are estimated by Cryo-SEM. The differentiation into lcd- and hcd-areas was less pronounced on the latter two sampling days, thus confirming the assumption that patches which are homogeneous in colour exhibit almost similar cell densities. The spatial microheterogeneity is not recognized by the LTT, which, however, allows sampling of larger sampling areas.

The cell densities and responses of the diatom assemblages of the three types of intertidal sediments differed significantly from one another. On May 7, IS-1 exhibited higher cell densities than IS-3 and IS-2, which were both of similar values. On May 20, IS-1 showed the lowest values whereas those of IS-3 and IS-2 had increased. On June 18, the three sediments exhibited almost similar cell densities. The overall values ranged from approx. 1000 up to 1,200,000 cells per cm^2 and were in the same order of magnitude as those calculated by other authors. Using Cryo-SEM, maximal cell densities of 40,000 and 1,000,000 cells per cm^2 have been reported for laboratory mesocosms [12, 13]. Field studies revealed values of up to 40,000 [11], 2,000,000 [1] and 200,000 diatom cells per cm^2 [15], respectively. For the laboratory tidal micro-ecosystem in which Paterson [12] investigated the vertical migration of diatoms, values up to 35,000 (+/– 6,400) cells per cm^2 were calculated.

VM has been described for several microorganisms, e.g. *Euglena obtusa*, *Tropidoneis lepidoptera*, *Hantzschia virgata*, for diatoms and dinoflagellates inhabiting the intertidal sands of the Visakhapatnam Beach, India, and for diatoms from the mudflats at Whitstable and from the banks of the river Avon, England [2, 5, 10, 11, 18, 19]. These time scheduled studies were done directly in the field or with sediment cores under laboratory conditions, and they clearly demonstrated the tidal/diurnal behaviour. Investigations on the tidal/diurnal VM by Cryo-SEM were restricted to a time scheduled study on a laboratory tidal micro-ecosystem which confirmed the data of those older field studies in general [12]. A standing population of cells in the first sample was taken at 6 am and at a photon flux density of 0.5 µM photons m^{-2} s^{-1} (equivalent to approx. 2.5 µM photons m^{-2} s^{-1} in the field). During the next 6 h of sampling, cell density increased 4-fold and finally, decreased in the last sample taken immediately before the tidal immersion of the mesocosm [12].

The reasons for the VM and the factors affecting this phenomenon are discussed controversely and might differ in different habitats. Round and Palmer [18] showed that VM was rhythmic and continued under constant illumination and temperature and removed from tidal influence. The finding that two species of *Euglena* and several species of diatoms expressed a diurnal (24 h) rather than tidal (24.8 h) period of the rhythms under laboratory conditions led [11] to the conclusion that all tidal VM rhythms actually represent underlying 24 h rhythms which are entrained and thus transformed by the tides in nature. The (24.8 h) tidal clock influences/superimposes an endogenous (24 h) biological clock. The authors postulated that VM could result from the interaction of a permanent positive geotaxis with a nonpermanent, rhythmic variation in phototaxis which can be modulated by the incident light. Thus, the cells generally perform a downward directed migration towards the sediment

bed. During the day they reach different physiological states and behave either photophobically (downward directed VM) or become attracted to light (upward directed VM). However, *Navicula* cf. *miniscula*, *Pleurosigma angulatum* and *Gyrosigma* sp. maintained a tidal rhythm in conditions of constant irradiance and temperature in the laboratory irrespective of the influence of the tides [6]. The VM was endogenous and remained tidal as the rhythm of vertical movement remained in phase with the tidal exposure of the field sites. Perkins, on the contrary, observed that littoral diatoms of the river Eden near St. Andrew, Scotland, did not show VM throughout the day, but stayed on the sediment surface with upcoming tide [17]. They were always submerged in the soil at night. The author concluded that the littoral diatoms of the river Eden estuary do not exhibit a tidal rhythm but a diurnal rhythm which is dependent on the intensity of light incident upon the shore. The waters of river Eden are clear and allow diatoms to perform photosynthesis even when being submerged. Paterson [12] registered that a significant proportion of cells did not show VM at all but stayed on the sediment surface throughout the entire time (most probably due to permanent positive geotaxis). Paterson [12] discusses light as a trigger controlling the onset of VM; different diatom species sense photon flux so that species specific thresholds have to be exceeded in order to induce VM. Thus, in the microtidal ecosystem examined species of *Nitzschia* and *Navicula* first migrated onto the sediment surface whereas *Scoliopleura tumida* came last. The downward oriented migration started with tidal immersion. The finding that the times at which the populations reached their maximum on the sediment surface varied between species has already been described [6, 7, 18]. Harper [7] registered the daily migration even in continuous darkness. This finding can be taken as a direct proof for an endogenous rhythm. The downward migration prior to tidal emersions was also found during our study on May 7. On June 18, we registered that the diatoms in the Wadden Sea sediments of Dangast buried back into the mud prior to inundation by the incoming tide. This observation matches the finding that a "*decrease in incident irradiance at dusk during periods of tidal exposure of the sampling sites* ***also*** *resulted in movement of the diatoms back into the sediment*" [6].

In the current study, sampling in each sediment was restricted to three dates. It was assumed that the cell densities of the first sampling would reflect the status before the onset of VM, i.e. increase of illumination on May 7 and the flow-off of water on May 20 and June 18. By the next sampling VM should have occurred. The last sampling should indicate whether upcoming tide (on May 7) or probably reduced photon fluxes (on May 20 and 18 June) had caused the reversion of VM. Our study partially confirms the findings of Paterson [12]. Generally, a large portion of cells seemed to stay on the sediment surface (see Fig. 2). Furthermore, it was registered that cells performed upward migration with increased photon flux or when the water drained off. However, downward migration with incoming tide was not registered in all sediments examined. Thus, cells that had performed upward migration stayed on the surface even with incoming tide or when it grew dusky (IS-3 on May 7, IS-1 and IS-2 on May 20). This finding is in line with the observations of Happey-Wood and Jones [6]. In nature, other, yet undefined parameters might influence the VM behaviour as well, thus superimposing and manipulating the effects of tidal water and photon flux. Wadden Sea sediments are inhabited by a complex community of aerobic, anaerobic, microaerophilic and aerotolerant chemo- and photoautotrophic microorganisms. Most of them live within distinct zones of the sediment bed. Thus, potential candidates which manipulate the VM behaviour of diatoms in nature are photosynthesis, salinity, transpiration, evaporation, micronutrient fluxes and waste products of aerobic and anaerobic processes which take place below the zone in which VM occurs. According to our findings, additional factors under natural conditions might be (i) the location of the sampled area relative to the high tide border line, (ii) sediment composition and (iii) permanent water coating. All of them might drastically influence the migration behaviour of diatoms. Currently, we cannot exclude that diatoms migrate back into the sediment beds after being submerged or during the night. Further field experiments with sampling over a period of 24 h are needed to elucidate this question.

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