

Limited sinking of *Phaeocystis* during a 12 days sediment trap study

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Abstract

There is a controversy discussion about the contribution of the genus *Phaeocystis* to the vertical carbon export with evidence for and against sedimentation of *Phaeocystis*. So far, the presence of *Phaeocystis* in sinking matter was investigated with methods depending on morphological features (microscopy) and fast degradable substances (biochemical analyses). In this study, we determine the occurrence and abundance of *Phaeocystis antarctica* in short-term sediment traps and the overlying water column during a 12-day time period in the Atlantic sector of the Southern Ocean with 454-pyrosequencing and microscopy counting. In the sediment trap samples, we only found few sequences belonging to *Phaeocystis*, which was not reflecting the situation in the water column above. The cell counts showed the same results. We conclude that *Phaeocystis* cells are not generally transported downwards by active sinking or other sinking processes.

Keywords: 18S rRNA gene, 454-pyrosequencing, microscopy, Southern Ocean

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Introduction

The Southern Ocean plays an important role for the sequestration of carbon dioxide (Sarmiento & Le Quere 1996). Climate warming is expected to lead to an alteration in the phytoplankton community structure towards small sized species such as haptophytes and prokaryotes (Falkowski & Oliver 2007; Daufresne *et al.* 2009; Li *et al.* 2009). Some modelling studies predict a decrease in carbon export when phytoplankton size declines (Cox *et al.* 2000; Bopp *et al.* 2001; Fung *et al.* 2005). Nevertheless, recent data show the sinking ability of small phytoplankton by forming packages (aggregates and colonies); thus, a shift to small cells may not necessarily lead to less carbon export (Amacher *et al.* 2013). In general, organisms with mineral shells or scales that also form large blooms, such as diatoms and coccolithophores, are thought to be the main contributors to the export of organic matter to the deep ocean (Klaas & Archer 2002). The 'naked' haptophyte species from the genus *Phaeocystis* (including the Southern

Ocean species *Phaeocystis antarctica*) also frequently forms large blooms in different regions of the world's oceans and constitute a key group for biogeochemical cycles and food webs (Schoemann *et al.* 2005). However, their contribution to carbon export at depth is still controversial (Reigstad & Wassmann 2007) due to the fact that they do not leave behind recognizable cell structures or stable biochemical signals in the sediments (Reigstad & Wassmann 2007). Wassmann *et al.* (1990) observed mass sedimentation of *Phaeocystis pouchetii* based on visual examination in the Barents Sea. Smith *et al.* (1991) suggested large-scale carbon export of *Phaeocystis pouchetii* in the Norwegian and Greenland Seas. In the Southern Ocean, *Phaeocystis antarctica*-derived carbon export was monitored via fluorescence and DMSP signals (DiTullio *et al.* 2000). Here, fast-sinking aggregates were supposed to enhance vertical export. In contrast, Gowing *et al.* (2001) and Pakhomov *et al.* (2002) did not detect significant export of *Phaeocystis antarctica* by examining long- and short-term sediment traps in the Ross and Lazarev Seas, respectively. In a *Phaeocystis*-dominated ecosystem in the North Sea, the carbon budgets suggested that sedimentation is of minor importance (Rousseau *et al.* 2000). Reigstad &

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Wassmann (2007) also concluded that vertical export of cell carbon originating from *Phaeocystis* is of minor importance.

Microscopy analysis of sinking matter depends on morphological features, while biochemical analyses often rely on fast degradable substances (e.g. pigments). Hence, such methods might not be appropriate to the identification of the small sized *Phaeocystis* cells and their remnants. Here, molecular tools such as 454-pyrosequencing overcome the drawbacks of conventional methods by tracing signals of DNA. This can be advantageous to reveal the presence of small sized protists, like single celled *Phaeocystis* spp., in deeper water layers and sediment traps. However, quantitative statements have to be handled with caution, because PCR biases and different gene copy numbers lead to over- or underrepresentation of certain species. Nevertheless, comparisons of relative abundances provide useful new insights into the variability and diversity of protist communities (Kilias *et al.* 2013, 2014; Wolf *et al.* 2013, 2014).

The objective of this study was to better understand the importance of *Phaeocystis antarctica* for vertical flux of carbon in the Southern Ocean. We determined occurrence and abundance of *Phaeocystis* in short-term sediment traps and the overlying water column during a 12-day time period in the Atlantic sector of the Southern Ocean. We applied 454-pyrosequencing of the V4 region of the 18S rRNA gene and microscopy counting. The hypothesis tested was that occurrence and abundance of *Phaeocystis* in the sediment traps reflect the

state from the water column above and around. Distribution of *Phaeocystis* sequences in combination with cell counts enables estimates on the sinking ability of this genus.

Materials and methods

Location and sampling

Sampling took place during the ANT XXVIII/3 expedition on-board RV *Polarstern* from 3rd to 15th of February 2012, in an eddy located at 12°S and 51°W. Water samples for molecular (1–2 L) and microscopy analysis (200 mL) were taken at the same location every third day (Fig. 1) at 20 and 100 m depth (additional samples at 60 m depth were taken at stations 91 and 140). Samples were collected using Niskin bottles attached to a Sea-Bird Electronics (SBE 911) CTD rosette. Further, sinking material was collected using free drifting arrays equipped with cylindrical sediment traps positioned to simultaneously sample fluxes at the base of the mixed layer (100 m or 120 m depth or 300 m or 320 m depth), above which most of the remineralization of sinking matter is likely to occur. At each depth, four cylindrical traps were deployed for about 1–3 days depending on the station. Subsamples from one cylinder filled with a nonpoisoned brine solution were used for molecular analyses and microscopy counts (1/8 split each obtained using a Folsom splitter). Sediment trap and water column samples for molecular analysis were

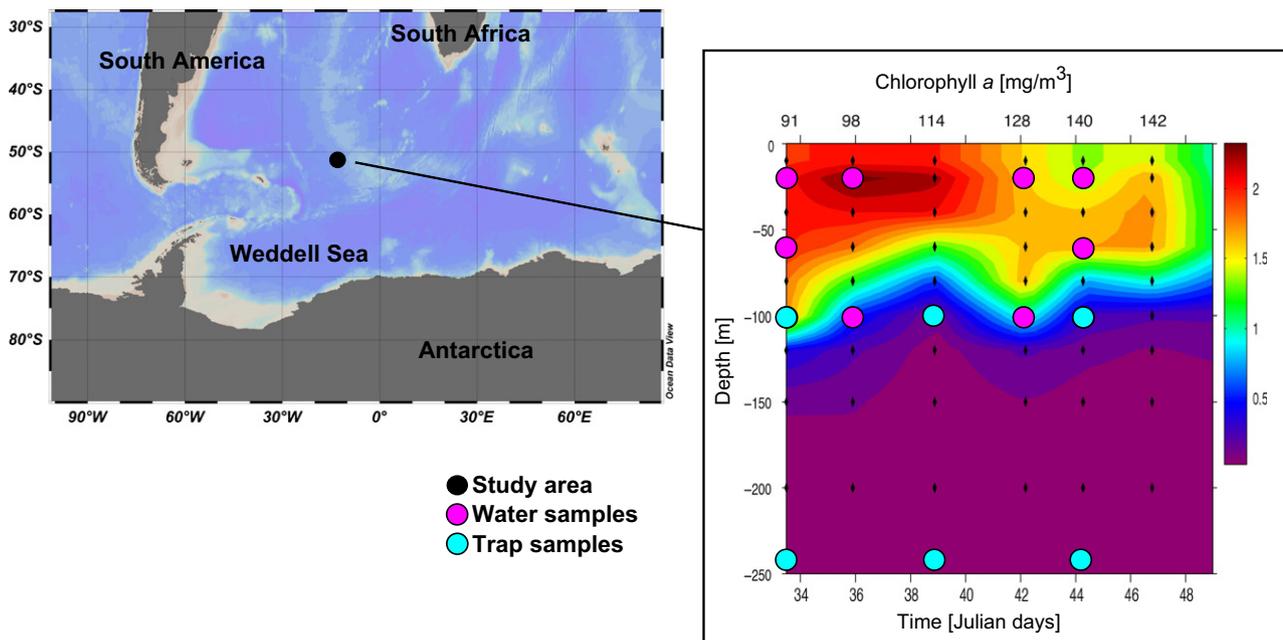


Fig. 1 Location of the study area and chlorophyll *a* plot showing the sample depth and sample day.

sequentially filtered through 10, 3 and 0.4 µm pore size Isopore Membrane Filters (Millipore, USA). Filters were immediately transferred to Eppendorf tubes and stored at -80 °C until analysis in the laboratory. The fractionated filtration facilitates the procedure itself and allows a separated amplification and thus minimizes the potential under-amplification of small protists, due to the lower gene copy number. Water column and sediment trap samples for microscopy were fixed with 2% final concentration hexamine-buffered formalin and stored in dark brown glass bottles at 4 °C until analysis.

Chlorophyll a measurement

Water samples for the determination of chlorophyll *a* (Chla) were obtained from the same bottles and depths as for the microscopy analyses. Chla samples were filtered onto 25-mm-diameter GF/F filters at pressures not exceeding 200 mbar. Filters were immediately transferred to centrifuge tubes with 10 mL 90% acetone and glass beads. The tubes were sealed and stored at -20 °C for at least 30 min and up to 24 h. Chla was extracted by placing the centrifuge tubes in a grinder for 3 min followed by centrifugation at 0 °C. The supernatant was poured in quartz tubes and measured for Chla content in a Turner 10-AU fluorometer. Calibration of the fluorometer was carried out at the beginning and at the end of the cruise. Chla content was calculated using the equation given in Knap *et al.* (1996) using average parameter values from the two calibrations.

DNA extraction, PCR amplification and 454-pyrosequencing

The DNA was extracted with the E.Z.N.A. TM SP Plant DNA Kit (Omega Bio-Tek, USA) as described in Wolf *et al.* (2014). For subsequent 454-pyrosequencing, the V4 region of the 18S rRNA gene was amplified with the primer set 528F (5'-GCG GTA ATT CCA GCT CCA A-3') and 1055R (5'-ACG GCC ATG CAC CAC CAC CCA T-3') [modified after Elwood *et al.* (1985)]. Details about the procedure, the PCR reaction mixture and the reaction conditions were described in Wolf *et al.* (2013). Each size fraction of each sample was amplified separately, and subsequent all three fractions of each sample were pooled before sequencing. Pyrosequencing was performed on a Genome Sequencer FLX system (Roche, Germany) by GATC Biotech AG (Germany).

Data analysis

Raw sequence reads were processed as described in Wolf *et al.* (2014) to obtain high quality reads. Briefly, reads shorter than 300 bp and longer than 670 bp, reads

with more than one uncertain base (N), chimeric reads, and reads belonging to metazoans were removed. Samples were subsampled to the lowest number of reads (here: 1454 reads). The remaining high quality reads of all samples were clustered (furthest neighbour algorithm) into operational taxonomic units (OTUs) at the 97% similarity level using the software Lasergene 10 (DNASTAR, USA). In the past, the 97% similarity level has tend to be the most suitable to reproduce original eukaryotic diversity (Behnke *et al.* 2011) and has also the effect of bracing most of the sequencing errors (Kunin *et al.* 2010). However, the similarity level is still under debate. The known intragenomic SSU polymorphism level strongly varies between different taxonomic groups. In dinoflagellate species, it can range up to 2.9% (Miranda *et al.* 2012). Consequently, different taxonomic groups may require different similarity levels, which hamper the choice for an appropriate similarity level for environmental samples. OTUs comprised of only one sequence (singletons) were removed. Consensus sequences were generated for each OTU, and their taxonomical affiliation was determined by placing them into a reference tree, containing about 1250 high quality sequences of Eukarya from the SILVA reference database (SSU Ref 111) (Quast *et al.* 2013), using the PhyloAssigner pipeline (Vergin *et al.* 2013). The compiled reference database is available on request in ARB-format. Numbers of reads belonging to *Phaeocystis* spp. were extracted for further analysis. The raw sequences were deposited at the Sequence Read Archive of the European Nucleotide Archive (ENA) under Accession no. PRJEB10928.

Cell counting via microscopy

Microscopy counting was carried out on a Zeiss AxioObserved D1 inverted epifluorescence microscope following the method of Utermöhl (1958) and recommendations of Edler (1979). Autotrophic protists were differentiated from heterotrophic protists by chlorophyll fluorescence. Before settling, 10 µL of a stock solution of the nuclear stain DAPI (Porter & Feig 1980) was added to the sediment trap sample, to help identification of protists alive at the time of sampling. Species-specific cell volumes were calculated from measurements and equivalent geometrical shapes following Edler (1979) and Hillebrand *et al.* (1999). *Phaeocystis* carbon biomass was estimated from counts and biovolumes as follows: cell numbers in colonies were estimated using the relation between colony volume (V_c in µm³) and cell number (N_c) given in Mathot *et al.* (2000), where $V_c = 417 \times N_c^{1.67}$. Carbon content of *Phaeocystis* cells in colonies was assumed to be 13.6 pg C per cells (Mathot *et al.* 2000). Further, in the case of colonies, the carbon

content of the mucous component was also estimated using the relationship C_c (ng C) = 213 V_c (ng/mm) of Mathot *et al.* (2000) and added to the carbon estimates for the *Phaeocystis* colonies. Carbon content (C_{sc} in pg per cells) of *Phaeocystis* single cells was estimated using the relationship of Menden-Deuer & Lessard (2000) with $C_{sc} = 0.216 \cdot V^{0.939}$, where V is cell volume in μm^3 .

Particulate organic carbon measurement

Water samples for particulate organic carbon (POC) analysis in the water column were obtained from the same bottles as those for Chla analysis and microscope counts. A split corresponding to $\frac{1}{2}$ content of a collection cylinder at each station and depth was processed for POC analysis in sediment traps. Trap and water column samples were filtered onto precombusted 25-mm-diameter GF/F filters at pressures not exceeding 200 mbar. Filters were immediately transferred to precombusted glass Petri dishes and dried overnight at 50 °C. Dried filters were stored at -20 °C until analysis in the home laboratory. Before analysis, samples were thawed at room temperature and a few drops of 0.1N HCl were added to the filters to dissolve the particulate inorganic carbon. Filters were then dried overnight (at 50 °C). Carbon content in filters was measured using a EuroVector Elemental Analyser. Measurements were blank corrected using measurements on filters taken during the cruise and processed as the samples using rinsing media. POC measurement variability based on measurements of three reference standards was 1.9%.

Statistics

For statistical analysis, data (sequencing and microscopy) were merged into three groups: 20/60 m, 100 m and Traps 100/300 m. No significant differences in *Phaeocystis* occurrence were observed between 20 and 60 m and 100 and 300 m sediment trap samples, justifying the pooling. The Shapiro–Wilk test was performed, revealing a non-normal distribution. A pairwise Wilcoxon rank sum test was performed to test for significant differences between the three groups. Statistical analysis was performed in R (R Core Team 2013).

Results

This study took place within a large-scale open ocean bloom at the boundary between the Polar Front (PF) and the Southern Antarctic Circumpolar Current south of the PF (Strass *et al.* in press). The bloom had started in mid-December (based on satellite observations, Hoppe *et al.* 2015; Fig. 1) and was in the declining phase during our sampling campaign, possibly due to

silicic acid and iron limitation as both nitrate and phosphate concentrations were high throughout the study period (Hoppe *et al.* 2015). The decline of the bloom prior and during our visit to the area occurred in a southeasterly direction, as a consequence, Chla standing stocks were lowest in the northwestern corner (55–60 mg Chla per m^2 and 10 g C per m^2 in the upper 100 m of the water column) and highest in the south-eastern corner (258 mg Chla per m^2 and 26 g C per m^2 in the upper 100 m of the water column) of our study area (see also Fig. 1 in Roca-Martí *et al.* 2016). Vertical fluxes showed an opposite trend with higher fluxes in the northwestern low biomass stations (0.9 and 0.2 g C per m^2/day at 100 m and 300 m depth, respectively) and lowest values in the southeast (0.4 g C per m^2/day and 0.1 g C per m^2/day at 100 m and 300 m depth, respectively), where highest phytoplankton and *Phaeocystis* cells and colony concentrations occurred. In general, we observed only few *Phaeocystis* colonies in the water column during the study. Single cells were representing the bulk of *Phaeocystis*. Although plankton biomass in the whole area was primarily dominated by diatom species (*Pseudo-nitzschia lineola* and *Dactyliosolen antarcticus*, Roca-Martí *et al.* 2016), *Phaeocystis antarctica* showed large changes in water column abundances with concentrations in the high biomass ‘low’ flux region (southeast) higher than in area with the lowest phytoplankton biomass and highest fluxes (northwest), in the range of 1.3–8.7 mg/m in the surface layer (10–20 m depth). Fluxes of *Phaeocystis antarctica* based on microscopy counts were low at all locations (between 0 and 0.17 mg C per m^2/day at 300 m depth).

Samples and trap deployments presented here were taken at a ‘central’ station close to the centre of a meandering structure in the area of study (Strass *et al.* in press), so as to minimize the effect of shear and advection. Our drifting trap deployments closely followed the trajectories of surface floats deployed to track surface currents (Strass *et al.* in press). The location was also in the middle of the observed geographical biomass and flux gradient described above (see also Fig. 1 in Roca-Martí *et al.* 2016) and was selected as potentially the best location to follow the temporal dynamics of particle export and composition in the collapsing bloom. In the upper layers at this location (20–60 m depth), Chla concentrations were always high ($>1 \text{ mg}/\text{m}^3$) but declined during the sampling period from $>2 \text{ mg}/\text{m}^3$ to $1\text{--}1.5 \text{ mg}/\text{m}^3$. At 100 m depth, Chla concentrations were always $<1 \text{ mg}/\text{m}^3$, except in one occasion at the beginning of the study where concentrations $>1.5 \text{ mg}/\text{m}^3$ were found. Chla measurements were carried out only down to 200 m depth. Values at this depth were well below $0.1 \text{ mg}/\text{m}^3$; this should also apply to 300 m depth, well below the Winter Water layer (Strass *et al.*

in press). During the study period, POC standing stocks in the central stations decreased from 195 mg C per m² to between 125 and 129 mg C per m² in the upper 100 m of the water column, while POC fluxes at 100–120 m depth and 300–320 m depth, respectively, almost equalled the upper water column standing stocks [average 149 ± 68 mg C per m²/day (±SD) and 81 ± 24 mg C per m²/day (±SD)]. These represent some of the highest fluxes measured in the Southern Ocean and indicate high turnover of material in the surface mixed layer (Roca-Martí *et al.* 2016).

Sequencing output

A total of 349 566 reads were retrieved from 454-pyrosequencing. After quality check, 97 809 high quality reads remained for further analysis. The amount of reads for each individual sample ranged from 1454 to 14 025. Therefore, samples were subsampled to the lowest number of reads (here: 1454 reads), resulting in 1300 *Phaeocystis* reads in total.

Phaeocystis abundances

The number of *Phaeocystis* sequences ranged from 7 (sample 128–100 m) to 266 (sample 140–20 m) in the water column and from 1 (sample 140–100 mST) to 10 (samples 114–100 mST and 140–300 mST) in the sediment traps (Fig. 2A). At 20 m depth, the number of

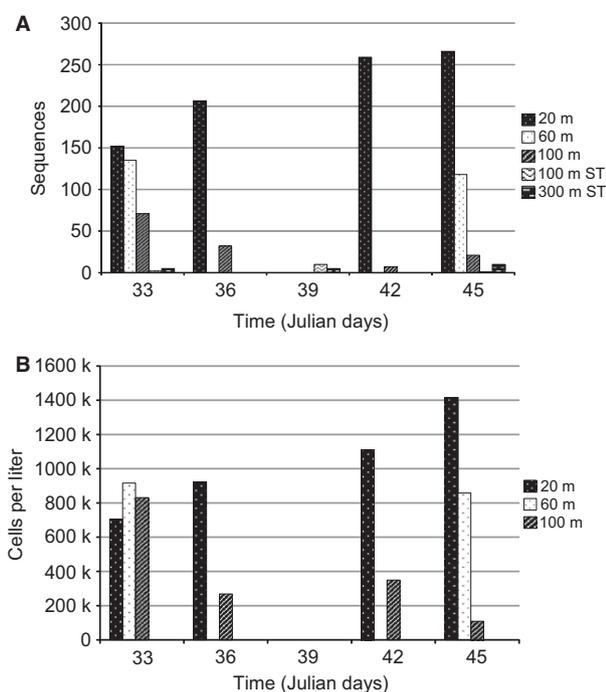


Fig. 2 Abundance of *Phaeocystis* (A) sequences and (B) cells during the 12 days period.

Phaeocystis sequences increased during the 12 days from 152 to 266, whereas at 60 m depth, the numbers were equal at the beginning and the end of the experiment. At 100 m depth, the amount of *Phaeocystis* sequences decreased during the sampling period. In the sediment trap samples, the number of *Phaeocystis* sequences never exceeded 10. The number of *Phaeocystis* cells (Fig. 2B) in the water column ranged from 108 567 cells/L (sample 140–100 m) to 1 415 622 cells/L (140–20 m). The cell counts showed the same trends as the numbers of sequences, that is an increase of *Phaeocystis* cells at 20 m depth, a constant amount at 60 m depth and a decrease at 100 m depth. The share of *Phaeocystis* cells in the total POC at stations 91 and 140 was significantly higher in the water column than in the sediment traps (Table 1).

Statistical analysis revealed significant differences (all *P*-values <0.05) in the number of *Phaeocystis* sequences and cell counts between 20/60 m, 100 m and in case of sequence data, the sediment trap samples (Fig. 3A,B).

Discussion

Our aim was to determine the occurrence and abundance of *Phaeocystis* in short-term sediment traps and the overlying water column, to better understand export of *Phaeocystis* on a short timescale. The hypothesis tested was that occurrence and abundance of *Phaeocystis* in the sediment traps reflect the state from the water column above and around. So far, to our knowledge, the sinking capability of *Phaeocystis* was not investigated by the use of DNA signals. Here, we present the first attempt to address this topic.

There is plenty of discussion about the contribution of *Phaeocystis* to the vertical carbon export. In our sediment traps, we only found few sequences and cells belonging to *Phaeocystis*, which was not reflecting the situation in the water column above. At 20 m depth, we observed an increase of *Phaeocystis* abundance over the 12 days period, while at 100 m depth, there was a slight decrease. The chlorophyll *a* values at 20 m depth were declining during the observation period, pointing to a fading of the phytoplankton bloom; however, this did not result in increasing export fluxes (POC). The decline

Table 1 Share of counted *Phaeocystis* cells in total POC flux. The total POC flux [mg C per m²/day] is given in parentheses (for 100 m water, the values are integrated)

Station	100 m water	100 m sediment trap	300 m sediment trap
91	2.08% (8532)	0% (111.4)	0.003% (71.8)
140	0.91% (15061)	0.1% (113.5)	0.13% (80.9)

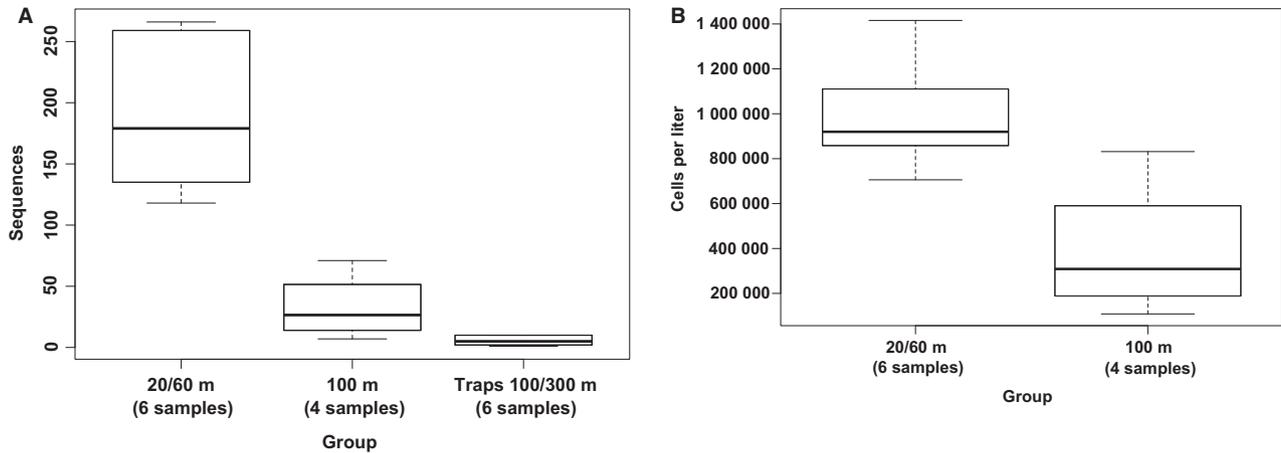


Fig. 3 Statistical comparison. (A) Number of *Phaeocystis* sequences in the three groups (20/60 m, 100 m, sediment traps). (B) Number of *Phaeocystis* cells in the two groups (20/60 m, 100 m).

of bloom forming species (e.g. diatoms) may account for the increase of *Phaeocystis*, which was reported to follow the diatoms during the succession (Grattepanche *et al.* 2011). The water samples at 100 m depth exhibited as far as 30-fold higher abundance of *Phaeocystis* sequences than the corresponding sediment traps. The contribution of *Phaeocystis* cells to the total POC was significantly lower in the sediment traps compared to the water column. These observations were confirmed by both of the applied methods, the sequencing and the cell counts. Thus, our hypothesis can be rejected. We conclude that *Phaeocystis* cells are not generally transported downwards in similar proportions as they occur in the water column by active sinking or other sinking processes. Another explanation for the limited observation of *Phaeocystis* in the sediment traps might be that they were especially exposed to grazing. The potential grazing pressure on sinking material has to be further evaluated. However, we expect to get roughly the same DNA signal with and without grazing, because DNA is relatively stable and is not digested within 24 h and still present in settling faecal pellets. There is not much work done on grazing on *Phaeocystis*. In general, flagellates sink within faecal pellets, but at low rates (see table 3 in Ploug *et al.* 2008). In contrast to our findings, mass sedimentation of *Phaeocystis pouchetii* based on visual examination was observed in the Barents Sea (Wassmann *et al.* 1990). Large-scale carbon export of *Phaeocystis pouchetii* in the Norwegian and Greenland Seas was suggested by Smith *et al.* (1991). In the Southern Ocean, *Phaeocystis antarctica*-derived carbon export was monitored via fluorescence and DMSP signals (DiTullio *et al.* 2000). Fast-sinking aggregates were supposed to enhance vertical export. In accordance with Passow & Wassmann (1994) and Riebesell *et al.* (1995), we observed no aggregate forming of *Phaeocystis* during

the 12 days period. It might be that special conditions (e.g. nutrients limitation, presence of grazers) trigger the formation of aggregates and we simply did not come upon these conditions. Consistent with our findings, Gowing *et al.* (2001) and Pakhomov *et al.* (2002) did not detect significant export of *Phaeocystis antarctica* by examining long- and short-term sediment traps via microscopy and pigment analysis in the Ross and Lazarev Seas, respectively. The carbon budgets in a *Phaeocystis*-dominated ecosystem in the North Sea suggested that sedimentation is of minor importance (Rousseau *et al.* 2000). Reigstad & Wassmann (2007) also concluded that vertical export of cell carbon originating from *Phaeocystis* is of minor weight.

Reigstad & Wassmann (2007) concluded that *Phaeocystis* spp. blooms are largely recycled in the upper layers, similar to our findings. Despite the high biomass and dominance of *Phaeocystis* spp. in pelagic systems, the vertical export of cell carbon is of minor importance (Reigstad & Wassmann 2007). This assumption is based on findings from the Barents Sea and North Norwegian fjords where there was a strong decline of *Phaeocystis pouchetii* cells between 40 and 100 m (e.g. Reigstad *et al.* 2000; Olli *et al.* 2002; Reigstad & Wassmann 2007), while vertical export of diatom cell carbon at 100 m was always larger than that of *Phaeocystis* spp. cell carbon (Reigstad & Wassmann 2007). We also observed a strong decline of *Phaeocystis* cells between 20 m and 100 m, at least after the first sample day.

Sediment traps may underestimate vertical flux of *Phaeocystis* spp. (Lalande 2008; Reigstad & Wassmann 2007) due to poor trapping efficiency. Thorium-234-based flux measurements during this study (Roca-Martí *et al.* 2016) showed, on average, a factor of about two times higher POC fluxes as compared to sediment traps. However, a direct comparison of Thorium-234 fluxes in

sediment traps compared to estimates based on water column values indicates only minor undertrapping (factors on average 1.7 ± 0.9 at 100 m and 3 ± 2 at 300 m depth) and possibly an overestimation of fluxes based on the Th-234 method resulting in higher Th-234 fluxes at 300 m than at 100 m depth (Roca-Martí *et al.* 2016, their table 3). However, if *Phaeocystis* was sinking actively or passively during the observed period, we would expect a larger DNA signal than we observed, because DNA is relatively stable and its detection does not depend on intact cells.

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C.W. designed research, performed research, analysed data and wrote the study. M.I. designed research and commented on the study. C.K. designed research, performed research and commented on the study. K. M. designed research, performed research and commented on the study.

Data accessibility

The raw sequences were deposited at the Sequence Read Archive of the European Nucleotide Archive (ENA) under Accession no. PRJEB10928.