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Climate-driven change in a Baltic Sea summer microplanktonic community – desalination play a more important role than ocean acidification

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Abstract

Scenario modeling suggests that the Baltic Sea, one of the largest brackish-water bodies in the world, could expect increased precipitation (decreased salinity) and increased concentration of atmospheric CO_2 over the next 100 years. These changes are expected to affect the microplanktonic food web, and thereby nutrient and carbon cycling, in a complex and possibly synergistic manner. In the Baltic Proper, the extensive summer blooms dominated by the filamentous cyanobacteria *Aphanizomenon* sp., *Dolichospermum* sp. and the toxic *Nodularia spumigena,* contribute up to 30% of the yearly new nitrogen and carbon exported to the sediment. In a 12 days outdoor microcosm experiment, we tested the combined effects of decreased salinity (from 6 to 3) and increased CO_2 concentrations (380 and 960 µatm) on a natural summer microplanktonic community, focusing on diazotrophic filamentous cyanobacteria. Based on our results, the most important factor was salinity, and pCO_2 showed only minor effects on total biovolumes of phytoplankton and abundances of heterotrophic bacteria. No interaction effects of salinity and pCO_2 were found on any of the measured parameters. The biovolume of the toxic *N. spumigena* was negatively affected by salinity 3, and the treatment with salinity 3 and 960 µatm CO_2 resulted in increased biomass of the presumably non-toxic *Dolichospermum* sp. Biovolumes of ciliates, diatoms and dinoflagellates were lower in salinity 3. Thus, the lower salinity seemed more important than increased pCO_2, and considering the Baltic Proper, we do not expect any dramatic effects of increased pCO_2 in combination with decreased salinity on the microplanktonic food web. We believe that our study can add one piece to the complicated puzzle to reveal the combined effects of increased pCO_2 and reduced salinity levels on the Baltic microplanktonic community.

1 Introduction

Climatic change is a global phenomenon affecting all ecosystems and a major societal concern since it may accentuate current anthropogenic effects (e.g. eutrophication), thereby changing and destabilizing the ecological balance of marine ecosystems. The Baltic Sea, one of the largest brackish-water bodies in the world, represents an ecosystem highly influenced by eutrophication, mediated especially by anthropogenic nutrient loading (Gustafsson et al., 2012; Kahru and Elmgren, 2014). Expected effects of climate change may compromise the Baltic Sea as a recreational and economical resource and there are strong indications that an increased frequency or duration of cyanobacteria summer blooms will cause serious harm to e.g. tourism industries (Hasselström, 2008), and additional impact on ecosystem health (Kabel et al., 2012).

Over the next 100 years, the Baltic Sea is expected to undergo a temperature increase by 2–5º C and experience increased precipitation rates, presumably leading to decreased salinity

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(HELCOM 2013). Concomitantly, the concentration of atmospheric CO_2 will increase from current values of ca 390 µatm up to >970 µatm by year 2100 (Meehl et al., 2007; IPCC, 2013). These dramatic changes will likely affect all trophic levels of the planktonic food web. Thereby, nutrient and carbon cycling will be affected in a complex and possibly synergistic manner, which may elicit ecosystem-wide regime shifts in higher trophic levels (e.g., Möllmann et al., 2009). The ecosystem is consequently pushed towards its natural limits, which may have serious ecological consequences (BACC Author Team, 2008). Therefore, it is pertinent to identify the ecological consequences of climate change in order to produce qualified predictions on the balance and function of the Baltic ecosystem in a future global change scenario. Due to the complexity of the systemic response, multi-factorial approaches rather than single-factor experiments (Havenhand, 2012; Lindh et al., 2012; Karlberg and Wulff, 2013; Eichner et al., 2014a; Riebesell and Gattuso, 2015) are required to decipher linkages between particular environmental changes and responses at various trophic levels of the food web.

In the Baltic Proper, the extensive summer blooms of cyanobacteria contribute up to 30% of the yearly new nitrogen and carbon exported to the sediment (HELCOM, 2007), and the blooms are dominated by the filamentous taxa *Aphanizomenon* sp., *Dolichospermum* sp. (formerly *Anabaena* sp.) and the toxic *Nodularia spumigena*. Cyanobacterial nitrogen is assimilated and transferred in Baltic food webs directly through grazing, or indirectly through bioavailable nitrogen exuded from cyanobacterial cells (Ploug et al., 2010, 2011; Karlson et al., 2015). Due to group-specific differences in carbon uptake and saturation states of photosynthetic rates, increased CO_2 concentrations will affect photosynthesis as well as phytoplankton community composition (Raven et al., 2005; Reinfelder, 2011). In laboratory experiments using filamentous Baltic cyanobacteria, here *N. spumigena*, effects of elevated CO_2 levels on growth ranged from decreased growth (Eichner et al., 2014b) to increased growth rate (Wannicke et al., 2012). However, lack of effects has also been reported for *N. spumigena*, *Aphanizomenon* sp. (Karlberg and Wulff, 2013) and *Dolichospermum* sp. (Brutemark et al., 2015). Theoretically, increased CO_2 levels should probably not affect heterotrophic bacteria directly (Joint et al., 2011), but direct effects with higher bacterial abundance at elevated pCO_2 have nevertheless been shown (Endres et al., 2014). Increased phytoplankton biomass or productivity mediatied by elevated pCO_2 may stimulate growth of particle-associated bacteria (Grossart et al., 2006; Engel et al., 2013). Hence, consequences of elevated pCO_2 levels for bacterioplankton are indeed difficult to predict.

Although the Baltic diazotrophic filamentous cyanobacteria seem to tolerate a wide salinity range, some differences between species have been reported. The toxic *N. spumigena* tolerated a salinity range of 5–30 with a biomass peak at salinity 10 (Lehtimäki et al., 1997) or 7 (Mazur-Marzec et al., 2005). *Aphanizomenon* sp. seems more sensitive to salinity changes, and the tolerance range was salinity 0–5 (Lehtimäki et al., 1997). However, *Aphanizomenon* sp.

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isolated from the Baltic Sea, has shown to be able to tolerate salinities up to 10 (Laamanen et al., 2002). *Anabaena* sp. (now *Dolichospermum* sp.) showed both higher growth rates and toxin concentrations at salinity 1–2 relative to salinity 5–6 (Engström-Öst et al., 2011). For heterotrophic bacteria, surface waters of the central Baltic Sea harbour members of typical freshwater bacterial groups and lack several typical marine taxa (Riemann et al., 2008; Herlemann et al., 2011). Hence, the estuarine/brackish local conditions have shaped a bacterioplankton community uniquely adapted to the local salinity regime.

The aim of this study was to test impacts of the A1FI (i.e. business as usual) scenario (Meehl et al., 2007) on a natural Baltic microbial community, focusing on the three dominating filamentous cyanobacteria species during the summer bloom. This scenario projects increased atmospheric CO_2 levels (from 380 to 960 µatm), and decreased salinity (by 3 units, here from 6 to 3) for the Baltic Proper area in 2100. In order to shed some light into the complexity of the systemic response, we studied interactive effects of pCO_2 and salinity in an outdoor experimental set-up with ambient radiation and temperature conditions.

2 Material and methods

2.1 Experimental set-up

The experiment was conducted between 16 and 28 July 2010 outside of Askö Laboratory (58° 49' N, 17° 38' E) in the Baltic Sea. A natural community of Baltic Sea pelagic microplankton, dominated by the cyanobacterium *Aphanizomenon* sp. was collected using plankton net (mesh size 25 µm). To avoid large grazers, the collected organisms were gently filtered through a 200 µm mesh. The microbes were inoculated in 0.2 µm filtered Baltic Sea surface water with either salinity 6 (ambient) or 3 (reduced) salinity, and divided into 4 l ultraviolet (UV) transparent Plexiglas aquaria (Mohlin and Wulff, 2009). Reduced salinity was obtained by diluting natural Baltic Sea water with Milli-Q water, and by compensating inorganic nutrient dilution by the addition of nutrients following N:P ratios of f/2 medium (Guillard, 1975). The aquaria were randomly placed in four basins filled with continuous flow-through seawater, exposing the microbes to natural fluctuations of temperature. Temperature in each basin was recorded with a logger (HOBO Pendant, Onset Computer Corporation, Bourne, USA). The basins were covered with green plastic mesh to reduce the irradiance, resulting in an approximate 60 % reduction of the photosynthetically active radiation (PAR, 400–700 nm). This reduction was equivalent to PAR intensities at water depths of 1–2 m in the surrounding water column at the sampling site, as measured with a LI-1000 datalogger equipped with a Li-COR UWQ5201 PAR sensor (Li-COR, Lincoln, USA). A PMA2100 radiometer equipped with a 2π PMA2132 PAR sensor and a PMA2110 UV-A sensor (Solar Light, Glenside, USA) was used to record irradiances under the mesh throughout the experiment.

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For each of the two salinity treatments, two partial pressures of CO_2 (pCO_2) were established by connecting each aquarium with a tube, constantly providing synthetic air (AGA Gas, Linköping, Sweden) with a pCO_2 of either 380 µatm (ambient pCO_2) or 960 µatm (enriched pCO_2). The gas was dispersed to the water by ceramic air diffusers at a flow rate of ~15 ml min^{-1}. For pCO_2 960 µatm, the effect of flow velocity (3, 9 and 15 ml min^{-1}) was tested in triplicate experimental aquaria over 4 days using a culture of *N. spumigena* with a cell density corresponding to total phytoplankton cell density at Day 0. Target pCO_2 was reached at 9 ml min^{-1} but 15 ml min^{-1} was chosen to compensate for an increased cell density over time. The aquaria were sealed with Plexiglas lids where small holes were maintained for gas outlet to prevent backpressure buildup. In addition, each aquarium was provided with a submerged tube connected to an external syringe, which was used to remove subsamples from the aquaria without opening the lids and, thus, disturb the pCO_2 of the headspace. Quadruplicated aquaria from each of the four treatments were subsampled around 08.00 a.m. at five occasions (Days 0, 3, 5, 9 and 12) during the experiment. In addition, between Days 10 and 11, subsamples were analysed hourly for 30 hours to study the diurnal cycle of pH and photosynthetic efficiency (Pulse Amplitude Modulated fluorometer, Walz Mess- und Regeltechnik, Effeltrich, Germany). After sampling, at Days 2, 5 and 9, 7 ml of f/2 medium without nitrate was added to every liter of remaining sample to maintain concentrations similar to the Baltic Sea's summer nutrient conditions. In an additional set of four aquaria manipulated with ambient levels, i.e. salinity 6 and 380 µatm CO_2, no nutrients were added and used as a control for nutrient enrichment. To observe whether there were biological or chemical processes changing the carbonate system, one additional aquarium per treatment was set-up without any microbes as a control. Because the aim of the experiment was to investigate potential combined effects of salinity and pCO_2, samples from "nutrient controls" are not included in statistical analyses but results from inorganic and particulate organic nutrien analyses, carbon chemistry and chl a are shown in Table 1–3. A field measurement of diurnal changes in pH and pCO_2 was performed between Days 4 and 5; samples were taken every second hour over 24 hours.

2.2 Phytoplankton species composition and growth

At Days 0, 3, 5, 9 and 12, 50 ml from each aquarium was preserved with alkaline Lugol's solution, stored in the dark and analysed within three months using the Utermöhl method according to HELCOM (2008). Half of the bottom surface of the chamber was viewed in 10x magnification (Axiovert 40CFL, micrometerocular 44 42 32 E-Pl 10x/20, Zeiss, Oberkochen, Germany) and all organisms larger than 30 µm was counted and grouped, either to species level or order. Length and width of filamentous species were also measured and biovolume (mm^3 l^{-1}) was calculated. In both 20x and 40x magnification, a diagonal of the chamber bottom was analysed and organisms including micrograzers (e.g. ciliates) no smaller than 8 µm were counted.

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Growth rate (mm^3 day^{-1}) for each cyanobacteria species was calculated separately for Days 0 to 3 and Days 9 to 12. The specific growth rate (μ day^{-1}) was calculated according to (ln *DB* – ln *DA*) / (*t*B – *t*A) where *D*A is the biovolume at the first day and *D*B the biovolume at the end of the period, *t*A as day A and *tB* as day B. For Day 12, species diversity was calculated by Shannon's index.

2.3 Photosynthetic pigments

At Day 12, 100 ml from each aquarium was filtered onto 25 mm GF/F filters (Whatman, GE Healthcare, Chicago, USA). Extraction and analyses followed Wright and Jeffrey (1997) and are described in detail in Mohlin and Wulff (2009). Pigments are expressed as concentrations (ng $cell^{-1}$). For aphanizophyll and 4-keto-myxoxanthophyll, the response factor for myxoxanthophyll was used.

2.4 Photosynthetic activity

Photosynthetic activity was estimated by variable chlorophyll fluorescence measurements in photosystem II (PSII) with a WATER-PAM chlorophyll fluorometer calibrated for cyanobacterial application (Walz Mess- und Regeltechnik, Effeltrich, Germany). Minimum fluorescence (F_0') was determined by applying a low level of light and the maximum fluorescence (F_m') by exposing the sample to a short saturation pulse of measuring light (>4000 µmol photons m^{-2} s^{-1} for 0.6 s). Variable fluorescence ($F = F_m' - F_0'$) and effective quantum yield ($\Delta F/F_m'$) were determined for all samples.

2.5 Bacterial abundance and production

Duplicate samples from each aquarium (1.5 ml) were fixed on Days 0, 3, 5, 9 and 12 with EM grade glutaraldehyde (Sigma-Aldrich, St. Louis, USA, 1% final concentration), and stored at -80 °C. Bacterial abundance was determined by flow cytometry (FACSCanto II, BD Biosciences, San Jose, USA) after staining with SYBR Green I (Molecular Probes, Thermo Fisher Scientific, Waltham USA, Marie et al., 1997) using 1.0 µm green fluorescent polymer microspheres (Duke Scientific Corporation, Thermo Fisher Scientific, Waltham USA) as internal standard in each sample. Fluorescent beads (True counts, Becton Dickinson, Franklin Lakes, USA) were used to calibrate the flow rate.

Bacterial productivity was measured by [^{3}H]-thymidine incorporation (Fuhrman and Azam, 1982) as modified for microcentrifugation by Smith and Azam (1992). From each aquarium, duplicate 1.7 ml aliquots were incubated in darkness with [^{3}H]-thymidine (20 nM final concentration, GE Healthcare, Chicago, USA) in sterile 2.0 ml capacity polypropylene tubes for ca. 1 h at *in situ* temperature. Samples with 5% trichloracetic acid added prior to the addition of isotope served as blanks. Thymidine incorporation was converted to carbon production using 1.4

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$\times 10^{18}$ cells mole^{-1} thymidine incorporated (average calculated from published Baltic Sea data, SE = 0.1 $\times 10^{18}$ cells mole^{-1} thymidine, n = 73, HELCOM guidelines, Helsinki Commission) and a carbon content per cell of 20 fg (Lee and Fuhrman, 1987). The appropriate use of 20 nM ^{3}H-thymidine was confirmed by saturation curves.

2.6 Stoichiometry and analyses of dissolved inorganic nutrients

For analyses of particulate organic carbon (POC), nitrogen (PON) and phosphorus (POP), at Days 0 and 12, 100 ml from each aquarium were filtered onto pre-combusted (400°C for 4 h) 25 mm GF/C filters (Whatman, GE Healthcare, Chicago, USA). The filters for POP analyses were washed with 0.1 M HCl and rinsed with Milli-Q prior to filtration. All filters were then frozen at -20°C and freeze-dried for 36 h (Heto Power Dry PL3000, Thermo Fisher Scientific, Waltham, USA). Filters for POC/PON analysis were grinded into a fine powder (MM301, Retsch, Haan, Germany) and analysed in a elemental analyzer (EA 1108 CHNS-O, Fisons Instruments, Thermo Fisher Scientific, Waltham USA) applying 2,5-bis-[5-ert.-butyl-bensoaxzol-2-yl]-thiophen as a standard. POP-filters were analysed at Tvärminne Zoological Station, Finland, according to Solorzano and Sharp (1980).

Samples for determination of inorganic nitrogen (DIN, NO_2^- and NO_3^-), phosphate (DIP, PO_4^{3-}) and silicic acid (Si) concentrations (µM) were filtered through 0.45 µm pore-size polycarbonate filters, frozen in -80° C until analysed using colorimetric determination performed on an autoanalyser (Grasshoff, 1999) at the accredited laboratory of the Swedish Meteorological and Hydrological Institute, Gothenburg (Sweden).

2.7 Determination of the carbon dioxide system

Samples for pH, and total alkalinity (A_T) were analysed following established protocols for seawater carbonate system determination (e.g. Dickson et al., 2007). A_T was determined by potentiometric titration (Metrohm 800 Dosino and Aquatrode with Pt1000, Metrohm, Herisau, Switzerland) in an open cell with 0.05 M hydrochloric acid (Mattsdotter et al., 2014). The precision of the A_T measurements was obtained by triplicate analysis of one sample, and was estimated to ca. ±3 µmol kg^{-1}. The accuracy of A_T was ±5 µmol kg^{-1} throughout the entire experiment and controlled using Certified Reference Material (CRM) supplied by A. Dickson (San Diego, USA). pH was determined spectrophotometrically (diode-array spectrophotometer, HP8452, Hewlett-Packard, Palo Alto, USA) on the total scale (pH_T) using a 2 mM solution of the sulphonaphtalein dye, m-cresol purple, as an indicator (Clayton and Byrne, 1993). Prior to analysis, the samples were thermostated to ~25° C and filtered through a 0.45 µm pore-size polycarbonate filter, to remove particles that could disturb the measurement. Samples were measured in a 1-cm cell, where the temperature was measured using a thermistor with a precision of 0.1° C. The analytical precision was estimated to ± 0.004 pH_T units, which was determined by a series of ten analysis of one sample. The pH_T of the indicator solution was measured daily in a

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0.2 mm quartz cell. The perturbation of seawater pH_T caused by the addition of the indicator solution was calculated and corrected for using the method described in Chierici et al. (1999). A_T, pH_T, salinity and temperature were used in a chemical speciation model (CO2SYS, Pierrot et al., 2006) to calculate pCO_2, total inorganic carbon (C_T), and pH_T at *in situ* temperature. We used the CO_2-system dissociation constants by Mehrbach et al. (1973) as refit by Dickson and Millero (1987).

2.8 Statistical analyses

Data were analysed with two-way ANOVA using SPSS software (PASW Statistics ver. 18, SPSS Inc., Chicago, USA) for each sampling day. Homogeneity was tested by Cochran's test and, where needed, data were transformed according to Underwood (1997). Significant differences were set as $p<0.05$. Correlations between concentration of heterotrophic bacteria and different species of microplankton including micrograzers (i.e. ciliates) were performed with Pearson correlation, using SPSS software as above.

3 Results

In our study, the most important factor was salinity, and pCO_2 showed only minor effects on total biovolumes of phytoplankton and biomass of heterotrophic bacteria. No interaction effects of salinity and pCO_2 were found. The biovolume of the toxic *Nodularia spumigena* was negatively affected by salinity 3, and during the ca 2 weeks experiment the initially dominating *Aphanizomenon* sp. was replaced by *Dolichospermum* sp. The A1FI scenario (salinity 3 and pCO_2 960 µatm) resulted in increased biomass of *Dolichospermum* sp. Although time was not considered a treatment factor for the experimental design, still some differences irrespective of treatment were apparent in the different variables measured ("Succession", see below).

3.1 Treatment effects

3.1.1 Biomass and community composition. No interaction effects were found but effects of salinity and pCO_2, respectively, were observed. On Day 12, total phytoplankton biomass, approximated by chl *a*, was significantly higher in salinity 3 compared to salinity 6 ($F_{(1,12)}=6.63$ $p=0.024$, Table 1). Phytoplankton composition differed between treatments; *Dolichospermum* sp. showed significantly higher biovolumes in treatments with salinity 3 compared to present day salinity of 6 (Fig. 1). The effect of lower salinity persisted throughout the experiment (Day 12; $F_{(1,11)}=10.40$ $p=0.007$). In addition, elevated pCO_2 stimulated the total biovolume of *Dolichospermum* sp., (Day 12; $F_{(1,11)}=5.26$ $p=0.04$), with the highest values found for the treatment with salinity 3 and elevated pCO_2 (Fig. 1). For the toxic *N. spumigena*, lower biovolumes were found in salinity 3 compared to salinity 6 (Day 12; $F_{(1,11)}=7.21$ $p=0.020$, Fig 1). The biovolumes of dinoflagellates and diatoms were negatively affected by reduced salinity and

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showed higher biovolumes in salinity 6 by Day 12 ($F_{(1,12)}$=8.86 p=0.012 (dinoflagellates); $F_{(1,12)}$=13.11 p=0.004 (pennate diatoms); $F_{(1,12)}$=33.04 p<0.001 (centric diatoms); Fig. 1). No significant treatment effects were observed for specific growth rate (μ day^{-1}) of cyanobacteria except for *Dolichospermum* sp., with initially (Days 0 to 3) lower growth rate in salinity 6 compared to salinity 3 ($F_{(1,11)}$=11.61 p=0.006). The highest growth rate of 1.2 day^{-1} was observed for *N. spumigena* between Days 9 and 12. Shannon's index showed that the highest biodiversity (1.40) was found at salinity 6 (Day 12; $F_{(1,12)}$=17.34 p=0.001).

The concentration of carotenoids confirmed the results from phytoplankton biovolumes with an overall dominance of pigments specific for cyanobacteria; myxoxanthophyll, canthaxanthin and echinenone. For these pigments, higher concentrations were found in salinity 3 compared to salinity 6 (Table 1). Fucoxanthin, here a proxy for diatoms, showed no treatment effects.

Total biovolumes of micrograzers were not affected by the treatments, but for ciliates higher biovolumes were found in salinity of 6 compared to salinity 3 (Day 12, $F_{(1,12)}$=5.24 p=0.041, Table 1). By Day 12, the biomass of heterotrophic bacteria was significantly higher in present day conditions of salinity 6 and pCO_2 380 µatm, respectively ($F_{(1,28)}$=5.20 p=0.030; $F_{(1,28)}$=7.39 p=0.011, Fig. 2). As a consequence of dilution to reach target salinity of 3, significant treatment effects were observed already at Day 0, with higher concentrations in salinity 6 ($F_{(1,28)}$=64.29 p<0.000). Neither bacterial productivity nor cell-specific productivity (Fig. 2) showed any treatment effects by Day 12, but initially both were higher at the present day salinity of 6 ($F_{(1,28)}$=45.32 p=0.000 and $F_{(1,28)}$=19.28 p=0.001, respectively).

3.1.2 Stoichiometry and dissolved inorganic nutrients. No significant treatment effects were observed for either concentrations or elemental ratios (POC, PON and POP, Table 2). Treatment effects were observed for DIN, DIP and Si concentrations (Table 2). Due to dilution, dissolved inorganic nutrient concentrations were higher in aquaria with salinity 6 at Day 0 (Table 2). At salinity 6, Si decreased at a higher daily rate between Days 5 to 9 compared to Days 3 to 5 ($F_{(1,11)}$ =26.54 p=0.0009). The Si decline was concomitant with increasing diatom biovolumes.

3.1.3 Carbon dioxide system. Initially (Day 0, before adding CO_2), pH_T in salinity 6 was 7.73 (SE 0.01) and in salinity 3, 7.70 (SE 0.01) (no significant treatment effects). From Days 3 to 12, the 960 µatm treatment had significantly higher pCO_2 compared to the 380 µatm treatment but differed from target pCO_2. At Day 12, pCO_2 for the 380 µatm treatment at salinity 6 was 403 µatm (SE 18) and for salinity 3, 342 µatm (SE 16). For the 960 µatm treatment, mean pCO_2 was 833 µatm (SE 108) at salinity 6, and 579 µatm (SE 39) at salinity 3. The continuous supply of CO_2 complicates interpretation of changes in pH in relation to treatments. Initial A_T was 1523 and 820 µmol kg^{-1} at salinities of 6 and 3, respectively (Table 3). The difference was due to the

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initial dilution performed to reach target salinity. At Day 12, A_T in the 380 µatm treatment was 1592 µmol kg^{-1} (SE 4) for salinity 6, and 962 µmol kg^{-1} (SE 4) for salinity 3 (Table 3). For the 960 µatm treatment, corresponding values were 1629 µmol kg^{-1} (SE 4) and 1020 µmol kg^{-1} (SE 4), for salinity 6 and 3, respectively (Table 3). The A_T increase was generally higher in the high CO_2 treatments, regardless of salinity.

3.2 Successional changes irrespective of treatment

3.2.1 Biomass and community composition. Changes with time were found in the structure of the microbial community, indicating a successional pattern during the experiment. For phytoplankton biomass, proxied by total cell biovolumes and chl *a* concentrations, a general decline from initial values was followed by an increase until termination of the experiment by Day 12 (Table 1, Fig. 1). However, total biovolume of dinoflagellates decreased from Days 0 to 12 (Fig. 1). The most striking result was the shift in cyanobacteria composition where *Aphanizomenon* sp. decreased from initial 87% to 15% and *Dolichospermum* sp. increased from 11% to 82% of the total filamentous cyanobacterial biovolumes. The successional pattern in phytoplankton biovolumes was mirrored by the concentration of heterotrophic bacteria, however, when phytoplankton biovolumes decreased the concentration of heterotophic bacteria increased (Figs. 1, 2). This trend was consistent until Day 9. Between Days 9 and 12 the biomass of both phytoplankton and bacteria increased. Bacterial productivity followed the bacterial biomass with the exception that cell-specific productivity differed from bacterial biomass and declined from Days 0 to 3 (Fig. 2). At Day 12, a significant correlation (Pearson, $r^2(15)=0.647$, $p=0.009$) between the concentration of heterotrophic bacteria and *N. spumigena* was found. An example of epiphytic bacteria associated to *N. spumigena* is shown in Fig. 3. There was no significant correlation between heterotrophic bacterial concentration and concentration of any other organisms, such as other cyanobacteria, dinoflagellates, diatoms or ciliates.

3.2.2 Stoichiometry and dissolved inorganic nutrients. POC and PON concentrations generally decreased over time while POP concentrations increased (Table 2). For all treatments, POC:POP and PON:POP were generally lower relative Redfield ratios of 106 and 16, respectively (Day 12). In contrast, POC:PON ratios were higher than the Redfield ratio of 6.6 for all treatments throughout the experiment (Table 2). The inorganic nutrient concentrations DIN, DIP and Si, decreased between Days 0 and 3 (all treatments, Table 2). DIN decreased drastically between Days 0 and 3, and remained low (<0.4 µM) throughout the remaining part of the experiment. DIP decreased the first three days of the experiment and increased by Day 5, leveling out Days 9 to 12. Si decreased continuously throughout the experiment, and at a faster rate towards the end of the experiment, in concert with the increasing diatom biovolumes. DIN:DIP at Days 3 to 12 was lower than the Redfield ratio of 16.

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3.2.3 Carbon dioxide system. During the experiment pH increased in all treatments (Table 3), indicating CO_2 uptake (net primary production) further supported by the decrease in pCO_2 for all treatments (despite the continuous supply of CO_2 to the aquaria). A_T increased in all treatments over time (Table 3). A_T is not affected by changes in pCO_2 and observed changes are likely caused by net assimilation of NO_3^- and H^+ (e.g. protein synthesis during photosynthesis).

3.3 Diurnal variations

3.3.1 Photosynthetic activity and pH in experimental aquaria. During the hourly measurements over 30 hours, no significant treatment effects were found in $\Delta F/F_m'$. However, all treatments showed dynamic response to radiation saturation where $\Delta F/F_m'$ was depressed from early morning to around 17:00 (solar time). From late afternoon the radiation stress diminished and $\Delta F/F_m'$ returned to original values (Fig. 4). Also a clear diurnal pH cycle was observed, with lowest values during night/early morning (7.57) and highest values in the evening (7.92) (Fig. 5).

3.3.2 Diurnal in situ variability of pH and pCO_2. Samples were taken every second hour for a 24-hour period directly *in situ* to observe the natural variability outside the aquaria. We found a clear diurnal cycle related to CO_2 uptake during photosynthesis, and pH varied from 7.79 early in the morning to 8.42 in the late afternoon (Fig 5). The opposite pattern was observed for pCO_2; the lowest value 118 µatm in late afternoon and the highest, 570 µatm, night/early morning. Mean A_T *in situ* was 1498 µmol kg^{-1} (SE 4).

3.3.3 Radiation and temperature. Intensities of PAR and UV-A during the experimental period are shown in Fig 6. Initially sunny conditions were followed by cloudy days from Days 7 to12. A sunny day, PAR in the water outside Askö laboratory measured 450 µmol photons m^{-2} s^{-1} at 1 meter depth, while cloudy days showed typical intensities of 180 µmol photons m^{-2} s^{-1}. Experimental temperatures followed fluctuations in ambient water surface temperatures.

4 Discussion

Our aim was to test the combined impact of decreased salinity (from 6 to 3) and elevated pCO_2 (from 380 µatm to 960 µatm), following the A1FI scenario (Meehl et al., 2007) on a natural Baltic Sea microbial community, focusing on filamentous cyanobacteria during the summer bloom. No interaction effects of salinity and pCO_2 were found but our results suggest that salinity is more important than pCO_2 as a driver of the microbial community structure and biomass. The biomass of the toxic *N. spumigena* was negatively affected by the reduced salinity, and during the experiment the initially dominating *Aphanizomenon* sp. was replaced by *Dolichospermum* sp. The shift between *Aphanizomenon* sp. and *Dolichospermum* sp. was also observed *in situ*. Heterotrophic bacteria seemed more affected by the phytoplankton biomass

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than the experimental treatments. The highest biodiversity (1.40, by Shannon's index) was found at salinity 6.

Both dinoflagellates and diatoms were negatively affected by lower salinity, thus, cyanobacteria like *Dolichospermum* sp. could get yet another competitive advantage in a future Baltic Proper. Our results suggest that the toxic *N. spumigena* will not grow as well in a lower salinity environment but, on the other hand, the increasing biovolume by Day 12 indicate acclimation. In laboratory studies, optimum salinity for growth of *N. spumigena* range from 7 (Mazur-Marzec et al., 2005) to 10 (Lehtimäki et al., 1997) but the species also grow well at both salinity 4 and 7 (Karlberg and Wulff, 2013). For *Aphanizomenon* sp., Lehtimäki et al. (1997) concluded that *Aphanizomenon* sp. preferred salinity 0 to 5 over salinity 10 and 30, and when comparing salinity 7 and 4, no effects on biovolumes of *Aphanizomenon* sp. were detected (Karlberg and Wulff, 2013). Elevated pCO_2 had a positive effect on total biovolume for *Dolichospermum* sp. with the highest values found in the treatment with salinity 3 and elevated pCO_2. In our study, no effect of the elevated pCO_2 was observed for *N. spumigena* and *Aphanizomenon* sp., contradictory to Eichner et al. (2014b), showing a decreased growth rate in elevated pCO_2 for *N. spumigena* and an increased growth rate at elevated pCO_2 by Wannicke et al. (2012). Moreover, Brutemark et al. (2015) reported that no effects on growth of *Dolichospermum* sp. were found when exposed to low pH/high CO_2. However, the latter three studies were performed on single-species cultures and as shown by Mohlin et al. (2012), under stressful conditions growth rate of *N. spumigena* was stimulated by the presence of *Aphanizomenon* sp., further complicating interpretations from single-species experiments. Micrograzers were not affected by pCO_2, which is consistent with results by Aberle et al. (2013) from a coastal planktonic community.

Presently, *N. spumigena* is the only species of the three dominating filamentous cyanobacteria species in the Baltic Proper that produces toxin. Freshwater species of both *Dolichospermum* sp. and *Aphanizomenon* sp. produce the neurotoxin anatoxin-a (Carmichael et al., 1975; Rapala et al., 1993) but not the Baltic species (Sivonen et al., 1990; Lehtimäki et al., 1997; Sellner, 1997; Laamanen et al., 2002). However, microcystin-producing *Anabaena* sp. is widespread in the Gulf of Finland (Halinen et al., 2007; Fewer et al., 2009). If salinity decreases and these freshwater species, or strains of species, invade the Baltic Proper, blooms might still be toxic, just with another kind of toxin than nodularin. The *Aphanizomenon flos-aquae* presently existing in the Baltic Sea is genetically homogeneous, suggesting that one genotype initially colonized the Baltic Sea (Barker et al., 2000) and little genetic variation opens up for other invading species or perhaps toxic strains of the same species. We measured nodularin on Days 0 and 10, according to Pattanaik et al. (2010), but concentrations were always below or very close to the detection limit of the instrument. It is therefore unknown whether the treatments had any

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effects on the toxin concentration. However, potential treatment effects on nodularin should be further investigated. The effects of salinity on cellular nodularin concentration varies, Mazur-Marzec et al. (2005) observed a positive correlation between salinity 0 and 35 but Lehtimäki et al. (1997) found the highest nodularin concentration at salinity 15 compared to both lower and higher salinities. In Pattanaik et al. (2010), the highest intra- and extracellular nodularin concentrations were observed under nitrogen limitation when shielded from ultraviolet radiation.

The specific treatment effects on heterotrophic bacteria (higher biomass in present-day conditions) are difficult to distinguish in our set-up where heterotrophic bacteria were not tested separately from the autotrophs. The abundance of heterotrophic bacteria was, for example, positively correlated to *N. spumigena* and a negative treatment effect on this species would, thus, negatively affect the associated bacteria. Furthermore, environmental conditions and treatments stressing the phytoplankton community could result in more dissolved organic carbon (DOC) available for the heterotrophic bacteria, leading to increased abundance (like we observed between Days 0 and 3). This was observed in a large ocean acidification study where the heterotrophic activity was closely coupled to the primary productivity and release of DOC (Engel et al., 2013). In another mesocosm study, Grossart et al. (2006) report indirect effects of pCO_2 on heterotrophic bacteria mediated by the phytoplankton community. On the other hand, Endres et al. (2014) found a direct stimulation of bacterial growth at elevated pCO_2 (lower pH). This stimulation was attributed to increased availability of gel particles as food source and substrate, plus enhanced enzymatic hydrolysis of organic matter. In the Baltic, however, Lindh et al. (2013) found that ocean acidification (lowering of 0.4 pH units) did not affect the biomass of a heterotrophic bacterial assemblage, neither alone or in combination with increased temperature but ocean acidification in combination with increased temperature resulted in a shift in the bacterial community composition. These results highlight the complexity of unraveling effects of climate change on natural microbial communities and further stress the importance of a multifactorial experimental approach. Moreover, salinity has been shown to affect both functional performance and composition of bacterial communities regardless of DOC composition (Langenheder et al., 2003). The heterotrophic bacterial assemblage in the Baltic Proper is typically an assemblage adapted to this brackish environment with a pronounced influence of freshwater groups and lack of typical marine species (Riemann et al., 2008; Andersson et al., 2010; Herlemann et al., 2011). A future less saline Baltic Proper could increase the proportion of freshwater groups with yet unknown consequences for the marine food web (e.g. Herlemann et al., 2011), however, Nydahl et al. (2013) suggest that in a future warmer and wetter climate, the heterotrophic bacterial activity will increase, with increased coastal hypoxia as a possible outcome.

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The highest biodiversity (by Shannon's index) was found at salinity 6, implying that a future Baltic Proper may host a lower phytoplankton biodiversity. Lower biodiversity generally implies a decreased resilience towards environmental (including anthropogenic) stress, however, if all species within a functional group respond similar to pressure a higher biodiversity will not offer resilience (Hughes et al., 2005). For the type of microbial communities studied, the close coupling between phytoplankton and heterotrophic bacteria further complicates interpretation of experimental treatment effects. For heterotrophic bacteria, the experimental treatments (salinity and pCO_2) from an ecological perspective could be considered press disturbance, where the shift in phytoplankton composition and biomass was comparable to pulse disturbance (Shade et al., 2012) with different implications for the heterotrophic bacterial community resistance and/or resilience (Baho et al., 2012; Shade et al., 2012).

In our study, A_T increased slightly during the experiment. Addition of CO_2 does not affect A_T but the exudation of organic substances containing basic functional groups could explain the observed pattern (cf Kim and Lee, 2009). Thus, to better describe the carbonate system other parameters such as dissolved inorganic carbon might be preferred in similar experiments (Gattuso et al., 2010; Schulz and Riebesell, 2013). To reach target salinities, seawater of higher salinity was mixed with Milli-Q water, thus reducing the buffering capacity of the experimental water. Consequently, the A_T of the experimental water differed from that of Baltic seawater of similar salinities. The reduced buffering capacity could lower pH but was not expected to affect the microorganisms in our experiment (cf Ploug, 2008; Karlberg and Wulff, 2013), moreover, the pH change *in situ* over 24 hr was between 7.79 and 8.42. In the aquaria, the diurnal variability in photosynthesis (i.e. carbon uptake) resulted in large variations in pCO_2 levels, despite the constant supply with CO_2-enriched air. Our measurements were performed in the morning and provide a snapshot of the carbonate system. Large variations of pCO_2 due to the diurnal cycle of primary production have also been observed in coastal surface waters (Borges and Frankignoulle, 1999; Fransson et al., 2004; Schulz and Riebesell, 2013). Despite the complexity, maintaining a constant pCO_2 in the medium lacks ecological relevance when performing CO_2 enrichment experiments on primary producers. Our experimental set-up generates a diurnal variable pCO_2, fluctuating with similar wavelengths as *in situ* conditions. Therefore, bubbling with CO_2 enriched air is an advantageous method when studying organisms capable of substantial CO_2 perturbation (Gattuso et al., 2010; Karlberg and Wulff, 2013; Torstensson et al., 2013, 2015).

Similar to our study, in a post-bloom Baltic microplankton assemblage no CO_2-related effects in neither inorganic nor organic N pool sizes, or particulate matter N:P stoichiometry were found (Paul et al., 2015). Although nutrient levels were low by Day 12, *in situ* nutrient concentrations (DIN, DIP) in the surface water at the samplings site were lower with typical values of 0.02–0.06 µM (DIN) and 0.02 µM (DIP). Our Si concentrations by Day 12, however,

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were generally lower compared to in situ values of 6–8.6 µM. Apart from our experimental treatments, the Baltic Proper is under pressure with increased internal loading of phosphorus, lowering DIN:DIP ratios which presumably benefit diazotrophic cyanobacteria (Wasmund, 1997; Vahtera et al., 2007a). In our study, nutrients without nitrogen and silicate were added to further mimic summer conditions in the Baltic Proper without introducing DIP limitation, and DIN:DIP ratios at Days 3 to 12 (<5) confirmed nitrogen limitation in all aquaria. However, also DIP decreased, which was most likely caused by the P-storing abilities of the cyanobacteria (Vahtera et al., 2007b; Mohlin and Wulff, 2009; Olofsson et al., 2016). Despite low concentration of Si, both cell numbers and biovolume of diatoms increased by the end of the experiment. It is worth to notice that the highest chl a concentrations by Day 12 was found in the treatment with salinity 3 and pCO_2 960 µatm, that is, a treatment with the lowest initial nutrient concentrations.

Consistent with our findings, results from the large mesocosm experiment in the Gulf of Finland 2012 (Hornick et al., 2016; Lischka et al., 2016) highlights the complexity of studying plankton community responses to increased pCO_2 levels. Considering the Baltic Proper, we do not expect any dramatic effects of increased pCO_2 in combination with decreased salinity on the microplanktonic food web. However, our study lasted 12 days, which can be considered a short time study. For example, in a laboratory study over 7 months, Torstensson et al. (2015) concluded that long-term acclimation was crucial for the diatom studied. To conclude with a remark from Riebesell and Gattuso (2015) with respect to ocean acidification research "The paramount challenge for our research community will therefore be to assimilate the growing knowledge in each of these diverging research branches into an integrated assessment of short- to long-term responses to multiple drivers and their underlying mechanisms at the level of organisms, populations, communities and ecosystems." Thus, we believe that our study can add one piece to the complicated puzzle to reveal the combined effects of increased pCO_2 and reduced salinity levels on the Baltic microplanktonic community.

5 Data availability

Data will be accessible according to the policy by Biogeosciences.

6 Author contribution

All authors have participated in planning and designing the experiment and interpreting data. A. Wulff, M. Karlberg, M. Olofsson, A. Torstensson and N. Ekstrand performed field work and laboratory analyses. F. S. Steinhoff and M. Chierici performed laboratory analyses. A. Wulff prepared the manuscript with contributions from all co-authors. The authors declare they have no conflict of interest.

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Table 1. Concentrations of photosynthetic pigments and biovolume of micrograzers. Pigment data are expressed as µg pigments l^{-1} for chlorophyll a (Chl a), fuxocanthin (Fucox), myxoxanthophyll (Myxo), canthaxanthin (Canthax), and echinenone (Echin). The biovolumes of two micrograzer groups (rotifers and ciliates) are expressed in mm^3 l^{-1}. The different treatments are four combinations of salinity (S6, S3) and carbon dioxide concentrations (C380, C960). Controls denote an extra treatment (S6 C380) without nutrient enrichment. Numbers show mean values of four replicate treatments and standard error is shown between brackets.

Day	Treatment	Chl a	Fucox	Myxo	Canthax	Echin	Rotifers	Ciliates
0	S6 C380	na	na	na	na	na	2.36 (0.30)	0.02 (0.01)
	S3 C380	na	na	na	na	na	2.46 (0.43)	0.06 (0.03)
	S6 C960	na	na	na	na	na	1.52 (0.11)	0.06 (0.04)
	S3 C960	na	na	na	na	na	1.82 (0.27)	0.03 (0.01)
	Control							
12	S6 C380	3.36 (1.47)	0.13 (0.05)	0.28 (0.13)	0.16 (0.06)	0.18 (0.08)	3.00 (0.48)	0.29 (0.07)
	S3 C380	5.68 (1.5)	0.22 (0.15)	0.88 (0.15)	0.23 (0.06)	0.43 (0.11)	2.25 (0.17)	0.25 (0.06)
	S6 C960	2.55 (0.25)	0.07 (0.02)	0.44 (0.23)	0.11 (0.01)	0.13 (0.04)	2.97 (0.33)	0.56 (0.09)
	S3 C960	8.71 (2.04)	0.10 (0.02)	1.03 (0.30)	0.33 (0.03)	0.64 (0.10)	2.59 (0.71)	0.24 (0.09)
	Control	1.06 (0.11)						

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Table 2. Concentrations of inorganic nitrogen (DIN, NO_2^- and NO_3^-), phosphate (DIP, PO_4^{3-}), silicic acid (Si), particulate organic carbon (POC), nitrogen (PON) and phosphorous (POP). All quantified data are expressed in µM. The different treatments are four combinations of salinity (S6, S3) and carbon dioxide concentrations (C380, C960). Controls denote an extra treatment (S6 C380) without nutrient enrichment. Numbers show mean values of four replicate treatments and standard error is shown between brackets.

Day	Treatment	DIN	DIP	Si	DIN:DIP	POC	PON	POP	POC:PON	POC:PON:POP
0	S6 C380	5.37 (0.22)	0.31 (0.02)	10.31 (0.28)	17.5 (1.19)	214 (17)	22.67 (2.18)	0.88 (0.03)	9.53 (0.63)	244:26:01
	S3 C380	3.05 (0.37)	0.27 (0.01)	5.33 (1.01)	11.5 (0.96)	114 (31)	12.28 (3.23)	0.86 (0.02)	9.16 (0.18)	134:14:01
	S6 C960	5.34 (0.06)	0.46 (0.03)	10.50 (0.54)	11.3 (0.60)	287 (59)	31.42 (5.58)	0.91 (0.02)	9.02 (0.47)	317:35:01
	S3 C960	3.06 (0.18)	0.26 (0.01)	5.84 (0.24)	12.1 (0.58)	112 (14)	12.41 (1.49)	0.86 (0.02)	9.02 (0.38)	132:15:01
	Control	5.43 (0.51)	0.29 (0.02)	11.52 (0.98)	19.5 (1.34)	176 (29)	21.03 (3.54)	0.88 (0.01)	8.39 (0.08)	199:24:01
12	S6 C380	0.20 (0.04)	0.08 (0.03)	0.61 (0.09)	2.75 (1.80)	106 (22)	11.64 (2.59)	1.45 (0.07)	9.20 (0.20)	74:08:01
	S3 C380	0.21 (0.04)	0.11 (0.02)	0.63 (0.17)	1.67 (0.52)	46 (13)	4.66 (1.34)	1.24 (0.08)	9.53 (0.23)	38:04:01
	S6 C960	0.18 (0.03)	0.23 (0.04)	0.77 (0.29)	0.75 (0.16)	151 (19)	15.99 (1.60)	1.46 (0.16)	9.39 (0.23)	108:11:01
	S3 C960	0.39 (0.15)	0.09 (0.02)	0.72 (0.16)	2.93 (1.02)	129 (68)	9.90 (2.71)	1.63 (0.20)	8.34 (0.07)	93:07:01
	Control	0.20 (0.02)	0.10 (0.03)	1.75 (0.77)	1.53 (0.34)	75 (23)	7.74 (2.33)	0.84 (0.05)	9.79 (0.29)	92:09:01

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Table 3. Experimental conditions and manipulations, including salinity, experimental temperature, total alkalinity (A_T) and pH total scale (pH_T). pH_T and A_T are measured and presented at 25°C. Data from day 0 were obtained before bubbling started. The different treatments are four combinations of salinity (S6, S3) and carbon dioxide concentrations (C380, C960). Controls denote an extra treatment (S6 C380) without nutrient enrichment. Numbers show mean values of four replicate treatments and standard error is shown between brackets.

Day	Treatment	Salinity	Temperature °C	A_T µmol kg^{-1}	pH_T
0	S6 C380	6.0	15.3	1523 (1.6)	7.59 (0.03)
	S3 C380	3.3	15.3	824 (5.6)	7.51 (0.03)
	S6 C960	6.1	15.3	1523 (3.2)	7.59 (0.04)
	S3 C960	3.2	15.3	815 (1.1)	7.44 (0.06)
	Control	6.0	15.3	1510 (0.4)	7.67 (0.01)
12	S6 C380	6.0	15.3	1592 (3.6)	7.92 (0.02)
	S3 C380	3.2	15.3	962 (3.8)	7.81 (0.02)
	S6 C960	6.0	15.3	1629 (3.6)	7.66 (0.05)
	S3 C960	3.2	15.3	1020 (4.2)	7.65 (0.01)
	Control	6.0	15.3	1582 (1.9)	7.87 (0.02)

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Figure legends

Figure 1. Biovolumes of diazotrophic cyanobacteria (a) and, (b) biovolumes of flagellates, green algae, diatoms and dinoflagellates. The different treatments are four combinations of salinity (S6, S3) and carbon dioxide concentrations (C380, C960). Vertical lines show standard error (n=4).

Figure 2. Bacterial cell numbers (a) and, (b) cell-specific bacterial productivity (CSP, estimated by thymidine uptake rates). The different treatments are four combinations of salinity (S6, S3) and carbon dioxide concentrations (C380, C960). Vertical lines show standard error (n=4). *NB:* Data points are positively nudged on the x-axis in order to properly display the error bars.

Figure 3. The filamentous cyanobacteria *Nodularia spumigena* with associated heterotrophic bacteria. Effects of climate change on the autotrophic community may have indirect effect on closely associated heterotrophic bacteria and their biogeochemical interactions.

Figure 4. Hourly measurements of effective quantum yield ($\Delta F/F_m'$) during 30 hours under ambient PAR (400–700 nm) conditions. Active down-regulation of photosynthesis is observed during midday trough $\Delta F/F_m'$ depression in all treatments. The different treatments are four combinations of salinity (S6, S3) and carbon dioxide concentrations (C380, C960). Vertical lines show standard error (n=4).

Figure 5. Variation in pH_T over 24 hours. The continuous bubbling of CO_2-enriched synthetic air provides a fluctuating pH during the diurnal cycle of primary production. The different treatments are four combinations of salinity (S6, S3) and carbon dioxide concentrations in µatm, (C380, C960) plus *in situ* sea surface values. Vertical lines show standard error (n=4).

Figure 6. Daily variations of photosynthetic active radiation (PAR, 400–700 nm) and ultraviolet-A radiation (UV-A, 320–400 nm) during the experimental period. The aquaria and light sensors were placed under a mesh to simulate light intensities experienced in the surface water of the Baltic Sea (see 2.1 Experimental design).

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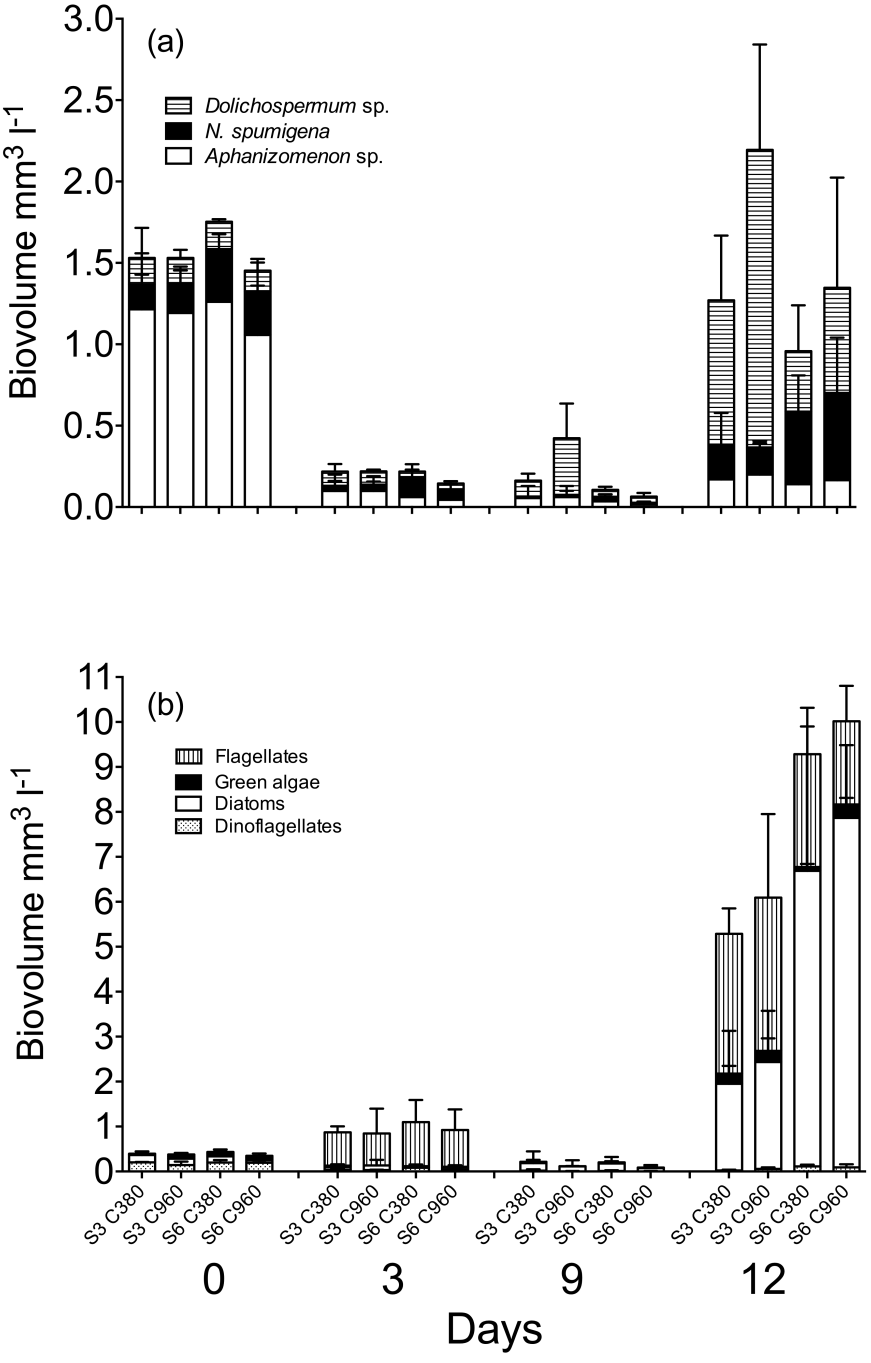

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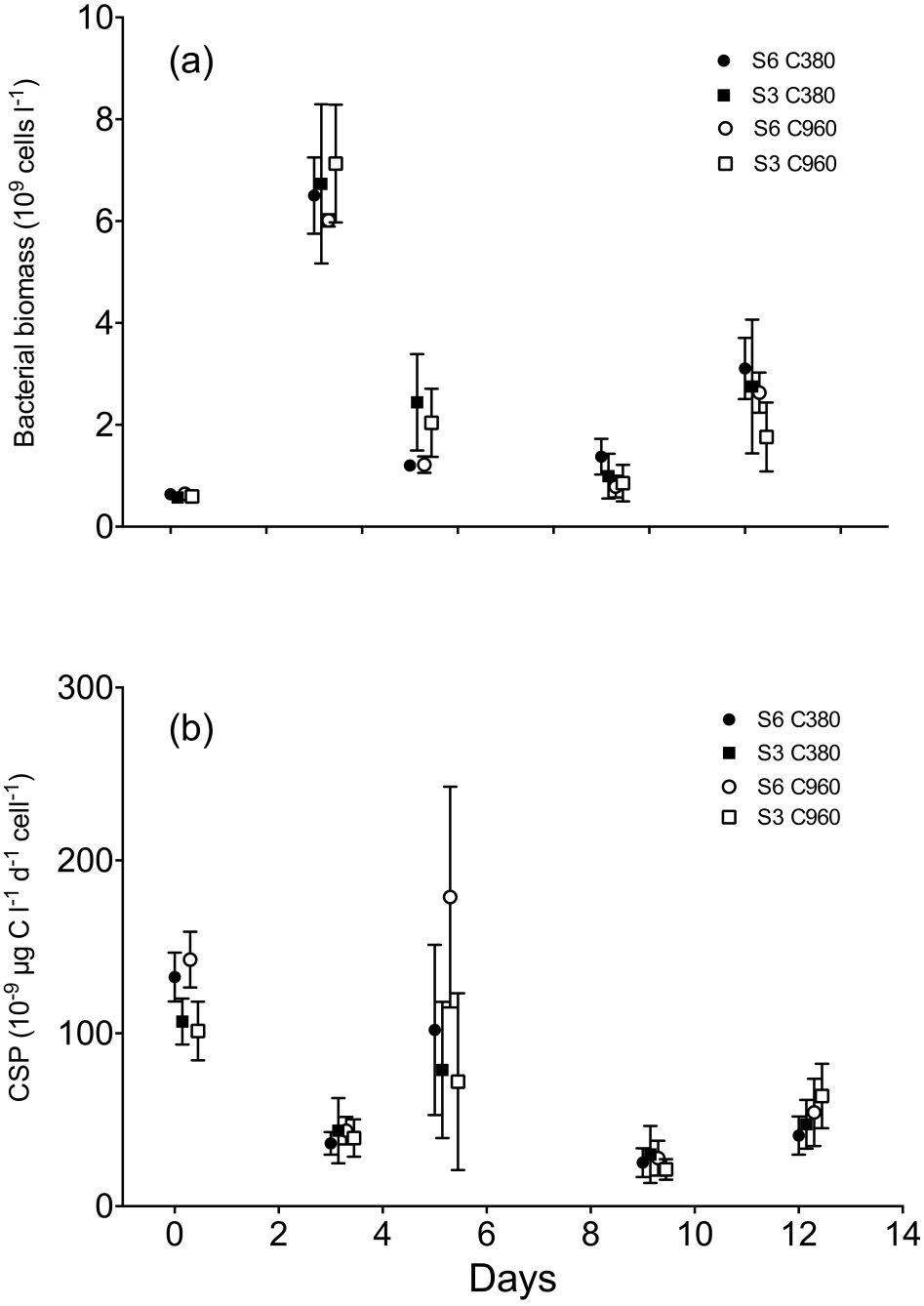

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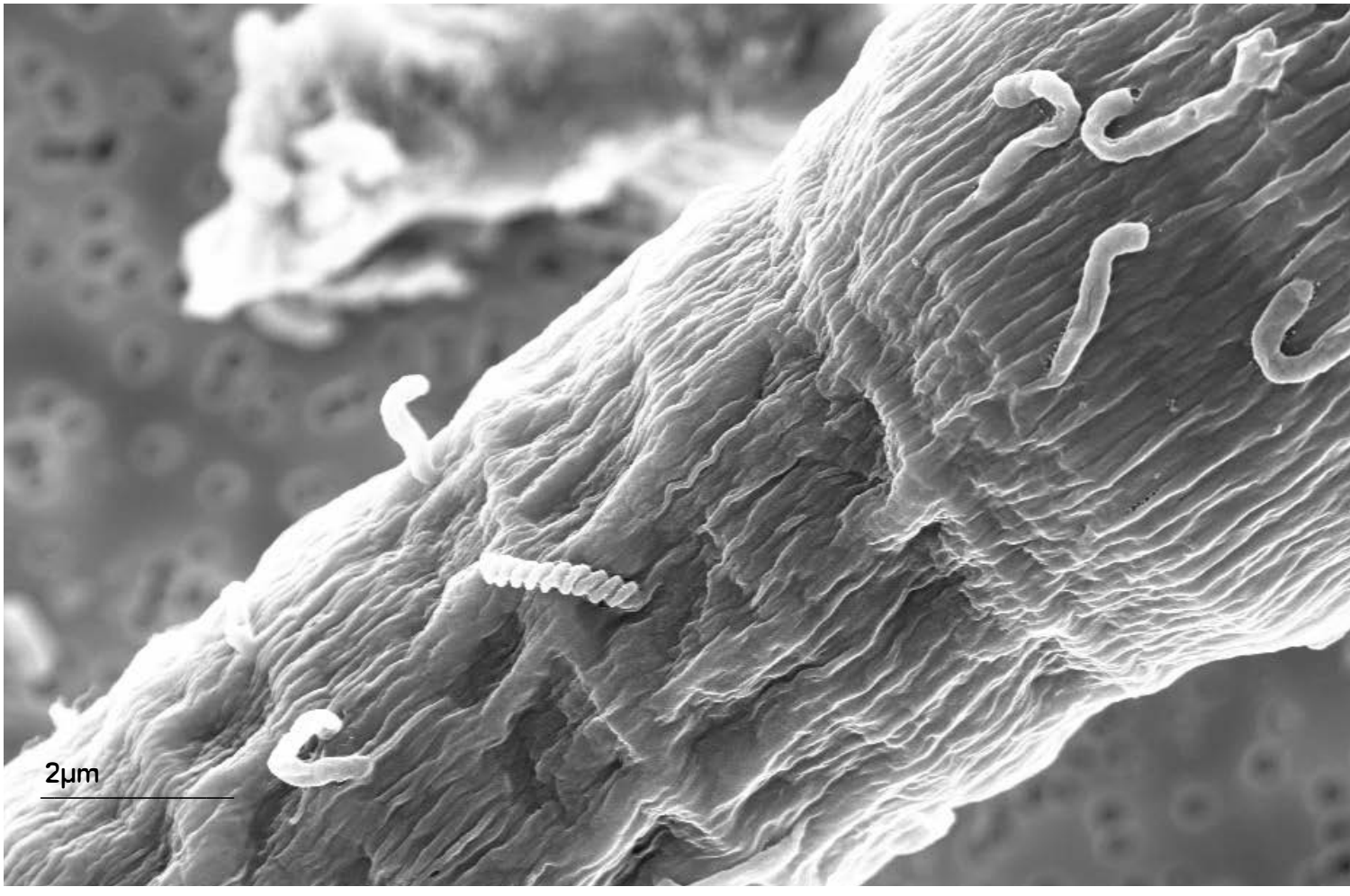

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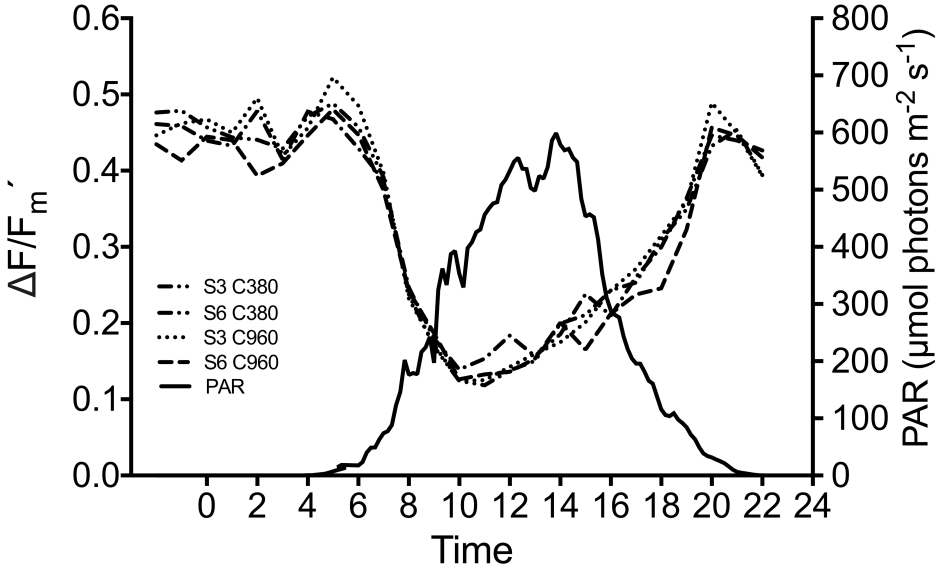

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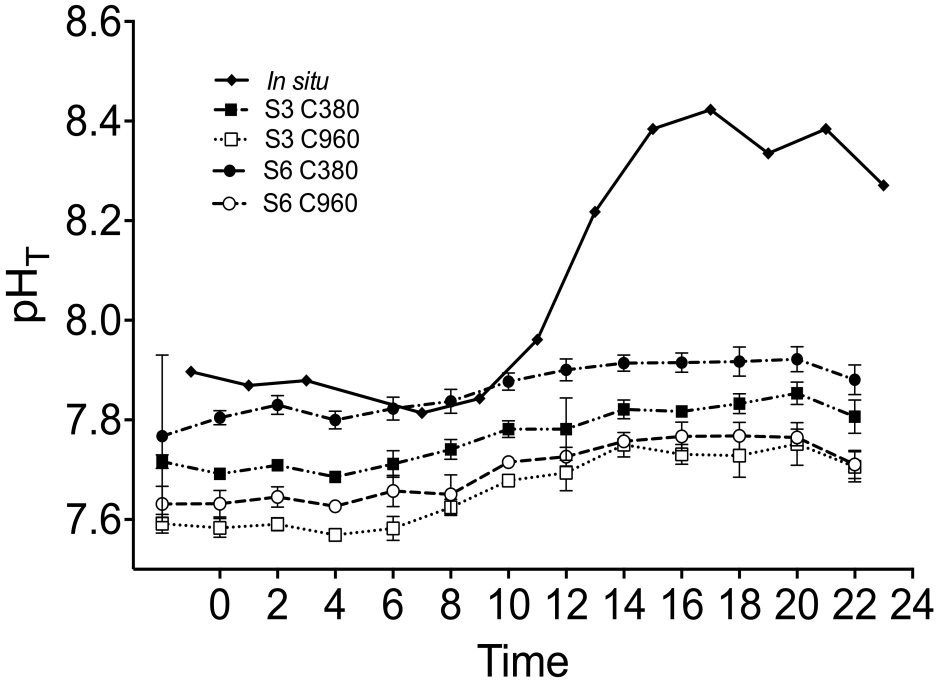

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