Material and methods BIOACID Experiment I, 2012

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Experimental design

Two different temperature regimes (i.e. 9 and 15°C) and two CO_2 levels (i.e. target values 560 ppm and 1400 ppm CO_2) were full-factorially manipulated using natural Baltic Sea phytoplankton assemblages in indoor mesocosms, each with a volume of 1400 L and a surface of 0.143 m³. Each treatment combination was replicated threefold. The resulting setup of twelve mesocosms was installed in four temperature controlled culture rooms.

Prior to experimental treatments mesocosms were filled with unfiltered natural seawater (salinity: 19.7) from Kiel Bight, Western Baltic Sea. The water contained the natural autumn community of phytoplankton, bacteria, and protozoa. Mesozooplankton from net catches (Kiel Bight) was added mimicking natural densities, i.e. 20 individuals l^{-1}. Each mesocosm was covered by a PVC cover containing a sampling port which remained closed between sampling events. After filling (19.10.12; hereafter called day -3), all mesocosms had similar temperature and CO_2 content. The following three days were used for applying the temperature and CO_2 manipulations and reaching divergence between the treatments levels.

To balance the natural draw down of CO_2 by phytoplankton production, over the course of the experiment CO_2 enriched water was added to the high CO_2 mesocoms at three times (29.10.12, 02.11.12, 09.11.12). The required volumes were calculated on the basis of DIC and alkalinity.

Over the course of the experiment, light was supplied by computer controlled light units (GHL Groß Hard- und Softwarelösungen, Lampunit HL3700 and ProfiluxII). Above each of the mesocosms one unit each consisting of 5 HIBay-LED spotlights (purpose build item of Econlux, each 100 W) was installed. Daily irradiance patterns

were constant over the experiment and computer controlled (GHL, Prometheus). The light-dark cycle was 11h50 min : 12h10 min.

Each mesocosm was automatically stirred by a gently moving propeller.

The experiment was ended after 24 days.

Sampling and measurements

Water temperature, salinity and pH were measured daily. Samples for phytoplankton biomass variables i.e. relative fluorescence, particulate organic carbon (POC), Chl a and phytoplankton carbon (microscopy and flow cytometer) as well as samples for POC (particulate organic carbon), carbon to nitrogen ratio (C/N), particulate organic phosphorous (POP) and particulate organic nitrogen (PON) were taken three times per week (Monday, Wednesday, Friday), which in total resulted in 10 samplings over the course of the experiment. Fatty acid samples were taken once a week (Friday).

Carbonate system - For measurements of total dissolved inorganic carbon (DIC) 10 mL samples were filled up into a glass vial (Resteck, Germany) using a peristaltic pump with a flow rate of 6 mL min^{-1}. The intake tube of the pump contained a single used syringe filter (0.2 µm, Sartorius). Filtered samples were poisoned with saturated $HgCl_2$ solution (20 µL), crimped with a headspace below one percent and stored dark at 4°C. DIC was measured following Hansen et al. 2013 using a SRI-8610C (Torrence, USA) gas chromatograph. For total alkalinity (TA) 25 mL samples were filtered (Whatman GF/F filter 0.2 µm) and titrated at 20°C with 0.05M HCl-solution (Dickson 1981, Dickson et al. 2003) in an automated titration device (Metrohm Swiss mode). The remaining carbonate parameter pCO_2 was calculated using CO2SYS (Pierrot *et al.* 2006) and the constants supplied by Hansson (1973) and Mehrbach et

al. (1973), that were refitted by Dickson & Millero (1987) and the KSO_4 dissociation constant from Dickson (1990).

Measures of phytoplankton biomass - Relative fluorescence was measured immediately after sampling using a fluorometer 10-AU (Turner Design). For chlorophyll a measurements, 250 mL water was filtered (Whatmann GF/F filters) and stored at -20°C until analyses took place. Prior to the photometrical measurements (HITACHI, U2900) filters were put into 8 mL acetone (90%) for 24 h in the dark at 6°C. Chlorophyll a content was calculated following Jeffrey and Humphrey (1975). Abundance of small phytoplankton (< 5 µm) was assessed by a flow cytometer (FACScalibur, Becton Dickinson) immediately after sampling, distinguished after size and pigment fluorescence (chlorophyll a and phycoerythrine). Larger phytoplankton (>5µm) were counted microscopically from Lugol fixed samples using an inverted microscope in Utermöhl chambers (Utermöhl 1958). Phytoplankton carbon was calculated by first converting cell abundances obtained from flow cytometry and microscopy to biovolume by multiplying cell numbers with linear measurements taking the nearest geometric standard (Hillebrand et al. 1999). Afterwards biovolume conversion into carbon content was done according to Menden-Deuer and Lessard (2000), i.e. $C= 0{,}288V^{0{,}811}$ for diatoms and $C= 0.216V^{0.939}$ for other phytoplankton (C=carbon content in pg, V=cell volume in µm³). As 180 µm³ is the smallest cell size included in the analysis of Menden-Deuer and Lessard (2000), their non-linear models predict unrealistically high C content for smaller algae. Therefore, conversion factors 0.108pgC/µm³ for diatoms and 0.157 pg C μm^{-3} for all other organisms were used for phytoplankton cells below 180 µ³ (Sommer et al. 2012).

Zooplankton sampling - Zooplankton was sampled weekly by three vertical net hauls, with hand held plankton net of 64 µm mesh size and 12 cm diameter. Each net haul sampled a volume of 5.1 L. All samples were fixed with Lugol's iodine. The total zooplankton catch was divided in a sample divider, so that ¼ of the total catch volume was counted and identified, developmental stages and sexes could be distinguished accurately. The prosome length constancy between molts enable a clear assignment of size at stage. All copepod were identified to genus level by using a ZEISS Discovery V.8 microscope with the magnification between 2.5x and 4.0x, and whenever possible developmental stage and sex of each individual were recorded.

Prosome length of identified copepods were digitally measured via photographs and digital software (ZEIS AxioVision 4.8 and AxioCam MRc) with a precision to the nearest µm. Means were calculated stage-specifically for copepods of each genus found in each mesocosm.

Single female adult copepods of the most abundant genus were collected individually from net catches on sampling days for stable isotope analysis, RNA/DNA analysis and only on start-and-end sampling for fatty acid analysis. 30 female adult individuals for fatty acid analysis, and 5 female adult individuals for RNA/DNA ratio were collected per mesocosm and sample and directly frozen in liquid nitrogen and stored at -80 °C until analysis.

For stable isotope analysis and C/N ratio, 30 female adult copepods of the most abundant genus of every sampling day were separated, washed in de-ionized water, and dried at 60 °C.

Particulate organic matter - For POC, PON, and POP 100-250 mL water (volume depending on plankton density) were filtered onto prewashed and pre-combusted

Whatman GF/F filters and immediately frozen in at -20°C. POC and PON were simultaneously determined by an element analyzer (Thermo Scientific Flash 2000). POP was measured colorimetrically following Hansen and Koroleff (1999) at 880 nm.

Fatty acids - For total fatty acid content and for fatty acid composition, filters with phytoplankton as well as tin cups with copepod samples, were extracted in chloroform / dichlormethane / methanol (1:1:1 v/v/v) following Arndt & Sommer 2013. Prior to extraction two internal standards, heneicosanoic acid (C21) and FAME mix (C19) were added. After extraction with n-hexane the fatty acid methyl esters were analyzed with a gas chromatograph (Thermo Scientific Trace GC Ultra with autosampler AS 3000). Peaks were identified by comparison with standard mixtures. For standardizing phytoplankton fatty acids to a biomass, they were related to POC.

Stable Isotope analysis – For stable isotope analysis copepod samples were dried at 50 °C and later on analyzed with a elemental analyzer (NA 1110, Thermo Electron, Milan, Italy) oven unit connected to a temperature-controlled gas chromatography (GC) oven (HRGC5160, Carlo Erba, Milan, Italy) and following stable isotops ratio measured in the isotope ratio mass spectrometer (Delta Plus Advantage, ThermoFinnigan, Bremen, Germany) after Hansen and Sommer (2007). Each sample run at 40 °C, with a dwell time of 5 min and then increased to 49 °C till the end temperature of 120 °C was reached. Sample gases were ionized in the mass spectrometer and finally the mass-to-charge ratio measured. The stable isotope data were expressed as delta values in parts per thousand (Hansen and Sommer 2007). The system was calibrated each measuring day by the combustion of International Atomic Energy Agency (N1-, N2-, N3-) and National Institute of Standards and

Technology (NBS-22) and graphite) compounds. Acetanilide was used as internal lab standard.

RNA/DNA ratio analysis - RNA-to-DNA ratio of pooled adult female copepods (5 individuals per sample) were measured following Speekmann et al. (2006) with the fluorescent dye RiboGreen, extraction buffer and microplate spectrofluorometer technique. RNA and DNA were measured using the cyanine base fluorescent dye RiboGreen, which binds non-specifically to nitrogenous bases of nucleic acids. RiboGreen and 20x TE buffer were purchased from (Sigma). RNA standard (type RiboGreen and 20x TE buffer were purchased from (). RNA standard (type III baker's yeast), DNA standard (type I calf thymus), RNase (tyoe III-A bovine pancreas), protease (type VIII bacterial), Triton X-100, and diethyl pyrocarbonate were purchased from Sigma.

Samples were homogenized in a shaking mill (Retsch Type MM-2) with 200 µl extraction buffer and glass beats in each sampling Eppendorf cap. All samples were shaken for 15 min. All steps were carried out on ice to minimize the possible effect of DNases and RNases. RNA and DNA standards were diluted in extraction buffer and determined using a ThermoFisher microplate spectrometer. DNA standards ranged from ~0.0 (blank) to 500 ng ml^{-1} and RNA standards ranged from ~0.0 (blank) to 1,500 ng ml^{-1}. 100 µl of copepod homogenate was placed in to black 96-well plates. RiboGreen reagent (100µl) was injected by hand into each well and afterwards shaken for 5 s, incubated for 5 min in the dark and initial fluorescent (FL_1) readings taken. RNase A (25µl) was then injected by hand into one set of standards, half of the blank wells, and all copepod samples, which were than incubated at 25 °C for 30 min. The final fluorescence value (FL_2) was then measured. FL_2 was a measure of present DNA, and FL_1-FL_2 equaled the amount of RNA present. Concentrations of

copepod RNA and DNA were calculated from RNA and DNA standard curves and RNA/DNA ratios were calculated from the pooled sample (5 ind per sample) to single individual amount.

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