Vol. 489: 93–105, 2013
doi: 10.3354/meps10438

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published August 28

Water nutrient stoichiometry modifies the nutritional quality of phytoplankton and somatic growth of crustacean mesozooplankton

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ABSTRACT: Here, we investigated how changes in water nutrient stoichiometry may change the nutritional quality of phytoplankton for mesozooplankton. For 6 d, we added nutrients to nine 1300 l mesocosms with natural summer phytoplankton from the Baltic Sea to create communities that were N-limited (P-treatment), Si-limited (PN-treatment) and not limited by nutients (PNSi-treatment). With the addition of P, no major changes occurred in phytoplankton biomass or species composition with time. With the addition of P and N, with or without excess of Si, C:N ratios approached the Redfield ratio, biomass increased and diatoms and dinoflagellates became dominant. Analysis of the 18S rRNA gene showed that the availability and diversity of phytoplankton other than diatoms and dinoflagellates, like prasinophytes or heterotrophic flagellates and ciliates, were not affected by the nutrient treatments. We then tested how the modified phytoplankton communities affected somatic growth in a natural mesozooplankton community dominated by the copepod genus *Acartia*. After 6 d of grazing, the zooplankton more than doubled their C content per individual when grazing on the Si-limited phytoplankton (PN-treatment) while total community biomass was maintained. In the other 2 treatments, the C content per individual remained the same and total community biomass decreased by ca. 60%, which suggests that the zooplankton was not optimally fed. Thus, a phytoplankton community with stoichiometry close to the Redfield ratio provided the best nutritional quality for the zooplankton, but not when the diatoms were Sisaturated. This study shows that nutrient stoichiometry in the seawater can affect zooplankton growth by modification of the phytoplankton community. It also shows that the food quality of phytoplankton does not depend only on the taxonomic composition, but also on the nutrient stoichiometry.

KEY WORDS: Eutrophication · Phytoplankton · Grazing · Mesozooplankton · Mesocosm experiment · Pigment composition · 18S rRNA gene

INTRODUCTION

Changes in inorganic nutrient ratios affect phytoplankton biomass and community composition, and may influence pelagic food-web dynamics (Olsen et al. 2006). In the brackish Baltic Sea, nutrient ratios vary between different basins and between coastal and open waters (HELCOM 2009). They also fluctuate with season and are affected by human activities. Eutrophication caused by N and P enrichment has been evident since the 1950s (HELCOM 2009). While N is the natural limiting nutrient for phytoplankton growth in most of the Baltic Sea (Granéli et al. 1990), Si may become limiting for diatom growth with increasing N and P levels (Conley et al. 1993). Si levels in the Baltic Sea have decreased during the last century, not only as a consequence of eutrophication but also through river regulation

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and increases in sedimentation of biogenic Si (Conley et al. 2008).

Si-limitation would likely shift the phytoplankton community composition from diatom-dominated to flagellate-dominated (Sommer 1994). A downward trend in spring and summer diatom abundance and biomass and a simultaneous increase in dinoflagellates have indeed been recorded in the Baltic proper since 1979 (Wasmund & Uhlig 2003, Klais et al. 2011). Also, chlorophytes, chrysophytes and cryptophytes have increased in some coastal areas during the last decades (Suikkanen et al. 2007), although large differences in phytoplankton trends occur between basins on a pan-Baltic scale (Jaanus et al. 2011). Blooms of filamentous cyanobacterial *Nodularia*, *Aphanizomenon* and *Anabaena* species are a natural feature in the Baltic Sea during N-limitation in summer, but may have increased in the last decades in some parts of the Baltic Sea (Suikkanen et al. 2007, Jaanus et al. 2011). The reasons for all these trends are not always clear, and may be linked to eutrophication and changes in nutrient stoichiometry intertwined with changes in climatic conditions (higher temperature, lower salinity) and stratification patterns (HELCOM 2009, Olli et al. 2011).

Mesozooplanktonic organisms (0.2 to 2 mm) are critical for the flow of matter and energy in the pelagic zone (Kiørboe 1997). In this study, we hypothesised that nutrient-induced changes in phytoplankton community composition and stoichiometry would affect the nutritional quality of the phytoplankton for the zooplankton. In a 2-stage experiment, we first added nutrients to mesocosms to create phytoplankton communities with different composition and stoichiometry (Expt I). We then tested how these modified phytoplankton communities affected somatic growth in a natural mesozooplankton community (Expt II). To assess phytoplankton community composition, we used 2 levels of taxonomic resolution. Phytoplankton pigment composition quantifies the taxonomic class level of the bulk of the biomass and indicates dominant groups such as diatoms and dinoflagellates (Mackey et al. 1996, Jeffrey & Vesk 1997, Paerl et al. 2003). As a complement to this, we used 18S rRNA gene analysis. The method we used fails to detect diatoms, but records other taxa that also may be important as prey for the zooplankton, such as small chlorophytes and heterotrophs (Moon-Van der Staay et al. 2001, Massana et al. 2002, Zhu et al. 2005). In the last decade, fingerprinting methods, have widely been used to describe the genetic diversity of phytoplankton communities in the field (Estrada et al. 2004, Yan et al. 2007), but experimental studies linking changes in genetic diversity to changes in environmental factors, like we used in this study, are still rare.

MATERIALS AND METHODS

Expt I (Day 1–7)

The first mesocosm experiment was carried out at the Zingst Biological Station (Germany), in the southern Baltic Sea (54° 25′ N, 12° 40′ E), from 31 August to 6 September 2001. The mesocosms consisted of 9 transparent polyethylene enclosures (height 2.2 m, diameter 0.9 m, volume 1300 l). The enclosures were submerged in the water with random placement, and attached to rubber rings on a floating raft with the upper edge of the enclosure about 40 cm above sea level. On 30 August 2001, the mesocosms were filled with natural seawater, which had been sieved through a 100-µm mesh plankton net, and left to stabilize overnight.

Three nutrient treatments were conducted in triplicate: (1) enrichment with P, (2) enrichment with P and N and (3) enrichment with P, N and Si. In the morning of each day, 0.25 µM NaH_2PO_4, 4 µM $NaNO_3$ and 12 µM Na_2SiO_3 were added to the appropriate mesocosms. These concentrations followed the Redfield ratio of N:P = 16:1 stipulated as optimal for phytoplankton growth (Redfield et al. 1963). The Si:N ratio added was about 3 times that necessary for optimal diatom growth of Si:N = 0.94 (Brzezinski 1985). Bicarbonate (cf. Redfield ratio C:N:P = 106:16:1), as well as trace metals (Fe, Mn, Zn, Cu, Co and Mo salts), vitamin B_{12}, citrate and biotin in 0.5% of f_2 medium concentrations (Guillard & Ryther 1962) were added to all 9 mesocosms daily. To avoid stratification, the mesocosms were stirred with a round plastic disc fixed to a rope 4 times each day: at 08:00 h before sampling, at 08:30 h after the nutrient additions, at 13:00 and 18:00 h.

Sampling took place in the morning of Day 1, Day 3 and Day 7 at 08:00 h. From each mesocosm, a water sample of 12 ml for inorganic nutrient analyses was filtered (0.45 µm) in the field and frozen at −20°C. Ten litres of water were sampled from each mesocosm and filtered through a 200 µm mesh plankton net. No mesozooplankton was observed on this net in the course of Expt I. For pigment, atomic C and N and molecular analyses of the phytoplankton, 50 to 400 ml of water (depending on the biomass) was filtered on GF/F Whatman™ glass fibre filters. All filters were immediately frozen in liquid N in the field and later stored at −80°C until analysis.

Expt II (Day 7–13)

The second mesocosm experiment was performed from 6 to 12 September 2001 using the 3 different phytoplankton communities created in Expt I. A wild mesozooplankton community dominated by calanoid copepods was collected in the morning of 6 September with a 200 µm mesh plankton net at 0 to 10 m of depth, about 1.5 km offshore. Within 1 h of sampling, the ca. 2 l net sample was diluted to a 50 l mesozooplankton stock suspension with filtered seawater, cleaned from macroscopic algae and dead and floating mesozooplankton, and added to each of the 9 mesocosms at 12:00 h. The zooplankton was starved for ~6 h prior to their inoculation to the mesocosms. Nutrient additions and stirring were conducted as in Expt I.

Phytoplankton samples and samples for inorganic nutrient analyses were taken as in Expt I on Day 9 and Day 13. Mesozooplankton from 5 to 7.5 l of water from each mesocosm was collected on Day 7, Day 9 and Day 13 on a 200 µm mesh plankton net on the same days at 12:00 h. The fraction <200 µm of this water was immediately poured back into its mesocosm. The zooplankton density in the mesocosms was later corrected for the fraction removed at each sampling occasion. The zooplankton samples on the net were transferred to 100 ml bottles with filtered seawater. One bottle per mesocosm was preserved with acid Lugol's solution for later analysis of the zooplankton community composition. On average, 566 zooplankton individuals per mesocosm were identified to genus and counted under a binocular microscope (Wild™ M5-26590) at ×50 magnification. The zooplankton from one bottle per mesocosm was collected on a GF/C Whatman™ glass fibre filter for atomic C and N analyses. All filters were immediately frozen in liquid N and stored at −80°C until analysis.

Temperature, salinity, pH and irradiation

On each day of both experiments, after stirring at 13:00 h, water temperature and salinity in the mesocosms were measured with a Yellow Springs Instruments™ TCS-meter, Model 33 and the pH was measured with a Metrohm™ Model 713 pH meter. Irradiation at the water surface was obtained from the weather station at the Zingst Biological Station (Germany), with measurements every minute, 24 h d^{-1}.

Dissolved inorganic nutrients

Dissolved inorganic nitrogen (DIN: NO_2^-/NO_3^--N and NH_4^+-N), dissolved inorganic phosphorus (DIP: PO_4^{3-}-P) and dissolved silica (DSi: SiO_4^{4-} -Si) were analyzed by segmented flow analysis using a Alpkem Flow Solution™ IV autoanalyzer.

Atomic composition

Particulate C and N on GF/F (phytoplankton) and GF/C (zooplankton) filters were determined with a Carlo Erba NA1500 elemental analyser (Carlo Erba Strumentazione). The organic material was combusted at high temperature and the combustion products were separated in a chromatographic column and analysed in a thermal conductivity detector.

Pigments

Chlorophylls and carotenoids were extracted from the phytoplankton samples on glass fibre filters using the method of Wright et al. (1997) as described by Andersson et al. (2003). For pigment separation, the reversed-phase HPLC method of Wright & Jeffrey (1997) was used with a slightly modified solvent system program (Pinto et al. 2003). Pigments were detected at 436 nm with a variable wavelength UV detector (Milton Roy™ SpectroMonitor 3100) coupled to a multiple solvent delivery system (Milton Roy™ CM 4000). For detection of phytoplankton pigments, a Spherisorb 5ODS (250 × 4.60 mm, 5 µm particle size) Phenomenex™ was used. For sample injection, an autoinjector (Spark Holland™, Promis II) was used. Pigment calibration for 19 pigments (Andersson et al. 2003), purchased from Roth™, Sigma™ and DHI Water and Environment™, was calculated using a 5-point calibration curve (with dilutions made in triplicate = 15 injections).

Nucleic acid extraction and amplification

One mesocosm within each treatment was randomly selected for the molecular analyses. Phytoplankton samples on GF/F filters were analyzed for Day 1, 3, 7, 9 and 13. Nucleic acids were extracted according to Tillett & Neilan (2000). The integrity of the total DNA obtained was verified by agarose gel electrophoresis. Nucleic acid extracts were stored at −80 °C until analysis.

PCR amplifications were performed using Hot start aq DNA polymerase (Hot Star, Qiagen™) and the universal 18S rRNA eukaryotic oligonucleotide primers EUK1A (Sogin & Gunderson 1987) and EUK516R (Amann et al. 1990; with a 40 nucleotide GC clamp at the 5′ end); and the chlorophyte-targeting 18S rRNA eukaryotic oligonucleotide primers CHLO02F (Simon et al. 2000; with a 40 nucleotide GC clamp at the 5′ end) and EUK1209R (Lim et al. 1993). These primers amplify ca. 516 and 464 bp, respectively. The PCR conditions followed Díez et al. (2001) for the universal 18S rRNA gene amplifications and a PCR annealing temperature of 50°C for the chlorophyte-targeting 18S rRNA gene amplifications. The PCR products were quantified with a Low DNA Mass Ladder (Gibco BRL) by agarose gel electrophoresis.

18S rRNA-DGGE

DGGE was carried out with a Dcode system (Bio-Rad™) to reveal the partial SSU rRNA genes from the eukaryotic part of the phytoplankton communities present in the samples with focus on chlorophytes. An equal amount of DNA (~800 ng) was loaded for each sample in the DGGE analysis. Electrophoresis was run at 75 V for 16 h in 0.75 mm thick, 6% polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) submerged in 1× TAE buffer (40 mM Tris, 40 mM acetic acid and 1 mM EDTA, pH 7.4) at 60°C as described by Díez et al. (2001). A linear gradient of denaturing agents, 45 to 65%, was performed for the universal 18S rRNA set of primers and 40 to 75% for the chlorophyte-targeting set of primers. After electrophoresis, the gel was first stained in 1 × TAE buffer containing SYBRGold Nucleic Acid Stain (1:10000 dilution; Molecular Probes™, Invitrogen AB) and the results were recorded using a molecular imager (ChemiDoc XRS system, BioRad™).

The DGGE banding patterns of the samples were compared by image analysis using the QuantityOne software (BioRad™) according to Schauer et al. (2000). The number of DGGE bands in each sample was interpreted as the number of phylotypes. The relative band intensity within each sample was interpreted as the relative abundances of the phylotypes in the sample. Presence or absence of individual DGGE bands in all lanes, as well as relative abundances (%) obtained, were taken into account to analyze community changes along treatments by PCA (see below). In addition, in order to obtain the identity of the DGGE bands, polyacrylamide fragments were excised from the gel using sterilized razor blades, resuspended in 20 µl of MilliQ water and stored at 4°C overnight. An aliquot of the eluted DNA was used for PCR reamplification with the same primers and conditions as above. The reamplified PCR products were cleaned using a PCR clean-up kit (GFX, Amersham™) and sequenced (with the corresponding forward primer) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), in an ABI PRISM model 377 (v.3.3) automated sequencer (DNA Technology).

Phylogenetic affiliations

The DGGE partial sequences obtained from 18S rRNA genes contained 500 and 405 bp for the universal eukaryotic and chlorophyte-targeting primers, respectively. These sequences were compared with the available sequences in public databases by performing BLAST searches at www.ncbi.nlm.nih.gov/blast (Altschul et al. 1997). All sequences were automatically aligned with the ClustalW alignment tool implemented in BioEdit™ version 7.0.4.1 (www.mbio.ncsu.edu/BioEdit/bioedit.html) and corrected manually to improve the alignment. Phylogenetic reconstructions were inferred using distance approximations by the neighbor-joining algorithm and the Kimura 2-parameters (K2P) correction, allowing gamma-distributed rates, in PAUP™ (version 4.0b10, Sinauer Associates). A total of 1000 bootstrap replicates were performed. The 18S rRNA sequences of *Euglena gracilis* and *Oriza* were used as outgroups. All sequences obtained in the present study were submitted to the GenBank database and are available under accession numbers: FJ949471 to FJ949484 (18S rRNA-PCR-DGGE bands EUK1-EUK13) and FJ949485 to FJ949495 (Chlorophytes18S rRNA-PCR-DGGE bands CHL1-11).

Statistical analyses

Repeated measures ANOVA's with post-hoc Tukey tests for the combined effects of treatment and day were performed with Statistica®, Version 10 (StatSoft). Principal components analyses (PCA) were performed with the programme CANOCO (ter Braak & Smilauer 2002) with log-transformed relative abundance data of pigments and DGGE bands as active variables (i.e. to construct the PCA ordination). Experimental treatment/day combinations were used as passive variables, i.e. they were fitted with

multiple regression analysis on the ordination results and not used to constrain the ordination itself.

RESULTS

Experimental conditions

During Expt I, temperature, salinity and insolation were slightly higher than during Expt II when the weather was cooler with more rain. During Expts I and II, water temperature (at 13:00 h) was 17.8 ± 0.5 and 15.4 ± 1.1°C (mean ± SD, n = 7 d), salinity was 8.5 ± 0.1 and 8.3 ± 0.1 and daily average irradiation was 98 ± 24 and 81 ± 24 µmol photons m^{-2} s^{-1} photosynthetically active radiation, respectively.

Expt I, nutrients

DIP did not become depleted in any of the 3 nutrient treatments (Fig. 1A). DIN was already low on Day 1 (0.4 µmol l^{-1}) and in the P-treatment it was nearly depleted after 6 d. However, in the PN- and PNSi-treatments, it increased (Fig. 1B). DSi was depleted in the PN-treatment after 6 d, remained the same in the P-treatment and became excessive in the PNSi-treatment (Fig. 1C).

The natural phytoplankton at Day 1 was heavily N-limited with a molar DIN:DIP ratio in the water of ca. 2 (Fig. 1D) and a molar C:N ratio of 8.8 (Fig. 1E). In the P-treatment, the DIN:DIP ratio decreased to 0.1 after 6 d, but in the PN- and PNSi-treatments it approached the Redfield ratio of 16 (Fig. 1D). In the P-treatment, the C:N ratio remained high after 6 d, but in the PN- and PNSi-treatments it decreased and approached the Redfield ratio of 6.6 (Fig. 1E). The Si:N ratio in the natural seawater on Day 1 was 15 ± 5 (mean ± SD, n = 9 mesocosms). The ratio increased to 40 ± 12 (n = 3) in the P-treatment and decreased in the PNSi-treatment (5.6 ± 1.4). In both cases, this is well above the Si:N ratio depicted for optimal diatom growth by Brzezinski (1985) of 0.94. However, in the PN-treatment, heavy Si-depletion occurred in the water with Si:N = 0.03 ± 0.01 on Day 7. During 6 d, the diatoms used up 2.0 ± 0.7, 8.6 ± 0.6 and 20.9 ± 4.1 mmol Si per 1300 l mesocosm in the P-, PN- and PNSi-treatments, respectively.

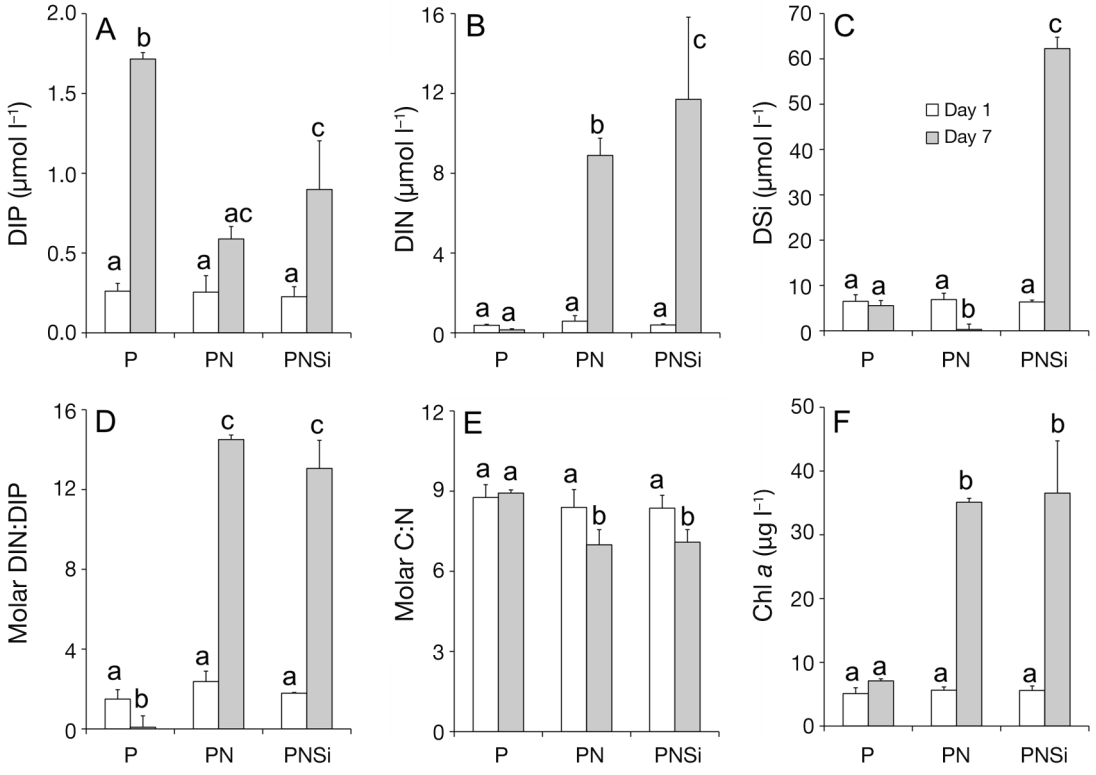

Fig. 1. Water nutrient concentrations, stochiometry and biomass on Day 1 and Day 7 in Expt I. (A) Dissolved inorganic phosphorus (DIP). (B) Dissolved inorganic nitrogen (DIN). (C) Dissolved silica (DSi). (D) Molar DIN:DIP ratio. E. Molar C:N ratio in the phytoplankton. (F) Phytoplankton biomass expressed as chlorophyll *a* (chl *a*) concentration per litre seawater. Different letters above the bars indicate significant ($p < 0.05$) differences for the combined effects of treatment and time tested with post-hoc Tukey tests in repeated measures ANOVA analyses

Expt I, biomass

As estimates of phytoplankton biomass, we use chlorophyll *a* (chl *a*) and particulate organic C concentrations per litre seawater. On Day 1, the biomass in the mesocosms was 5.4 ± 0.5 µg chl *a* l^{-1} and 1.3 ± 0.2 mg C l^{-1} (mean ± SD, n = 9 mesocosms). In the P-treatment, the chl *a* concentration was still low after 6 d, but in PN- and PNSi-treatments it increased almost 5 times (Fig. 1F). Similarly, C concentrations stayed the same in the P-treatment, but increased in the PN- and PNSi-treatments to 2.0 ± 0.6 and 2.0 ± 0.3 mg C l^{-1} (mean ± SD, n = 3 mesocosms), respectively. The pH in the PN- and PNSi-treatments increased to 8.5 and 8.7 at Day 7, respectively, but stayed at the natural pH of 8.1 in the P-treatment. This indicates a higher primary production in the PN- and PNSi-treatments through photosynthetic C uptake.

Expt I, community composition

The initial phytoplankton pigment composition (Table 1) was dominated by the chlorophylls *a* (43.1%), *b* (8.4%) and *c* (6.7%), and the carotenoids fucoxanthin (10.0%), zeaxanthin (5.4%), lutein (4.1%) and alloxanthin (4.7%). This pigment composition signifies a natural phytoplankton community consisting of diatoms (chl *c*, fucoxanthin), chlorophytes (chl *b*, lutein, zeaxanthin), cryptophytes (alloxanthin), prymnesiophytes (19′ hexanoyloxyfucoxanthin) and dinoflagellates (peridinin).

The PCA ordination of the pigment analyses (Fig. 2A) showed that substantial changes in phytoplankton community took place as a result of the nutrient treatments. The eigenvalues of the first 2 ordination axes were 0.68 and 0.11, respectively, which demonstrates that, by far, most of the variation

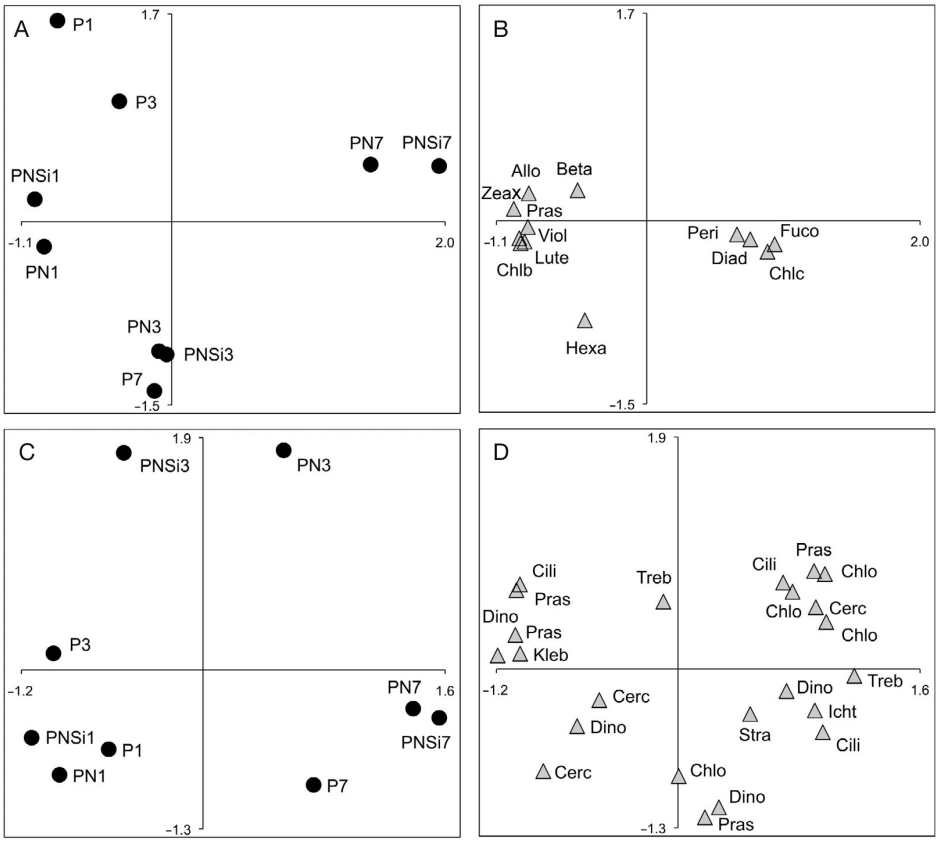

Fig. 2. PCA ordination plots showing changes in phytoplankton composition during Expt I. (A) Centroids for treatment/time combinations in the PCA analysis with the pigments (eigenvalues: Axis 1 = 0.68, Axis 2 = 0.11). (B) Scores for the pigments in the same PCA analysis as A (Allo = alloxanthin, Beta = beta-carotene, Chlb = chlorophyll *b*, Chlc = chlorophyll *c*, Diad = diadinoxanthin, Fuco = fucoxanthin, Hexa = 19′ hexanoyloxyfucoxanthin, Icht: ichthyosporea, Lute = lutein, Peri = peridinin, Pras = prasinoxanthin, Viol = violaxanthin, Zeax = zeaxanthin). (C) Centroids for treatment/time combinations in the PCA analysis with the 18S rRNA phylotypes (eigenvalues: Axis 1 = 0.46, Axis 2 = 0.21). (D) Scores for the phylotypes in the same PCA analysis as C (Cerc = Cercozoa, Chlo = Chlorophyceae, Cili = Ciliophora, Dino = Dinophyceae, Kleb = Klebsormidiophyceae, Pras = Prasinophyceae, Stra = tentatively identified as heterotrophic stramenopiles, Treb = Trebouxiophyceae)

Table 1. Changes in the composition of the zooplankton community in percent of total number of individuals (mean ± SD), n = number of mesocosms. Other zooplankton was <0.1%

	n	*Acartia*	*Eurytemora*	*Temora*	*Evadne*
Day 7	9	66.7 ± 5.7	5.9 ± 0.8	0.8 ± 0.3	26.6 ± 5.3
Day 13					
P-treatment	3	61.3 ± 4.3	11.2 ± 1.0	0.9 ± 0.2	26.6 ± 5.2
PN-treatment	3	52.1 ± 10.1	5.3 ± 0.3	0.1 ± 0.1	42.5 ± 10.4
PNSi-treatment	3	70.4 ± 11.1	4.8 ± 1.0	0.2 ± 0.2	24.6 ± 10.3

in the data set was explained by the PCA Axis 1. For Day 1, the centroids of all treatments were placed to the left of Axis 1, which describes the initial community (Fig. 2A). After 6 d, all centroids of the P-treatment were still to the left of Axis 1, indicating that only small changes in community composition had taken place. In the PN- and PNSi-treatments, large community changes occurred after 6 d as shown by the centroids to the far-right of Axis 1. These large changes were associated with relative increases of microalgae containing chl *c*, fucoxanthin, diadinoxanthin and peridinin (diatoms and dinoflagellates) and decreases of microalgae containing chl *b*, lutein, violaxanthin, prasinoxanthin and zeaxanthin (chlorophytes), alloxanthin (cryptophytes) and 19′ hexanoyloxyfucoxanthin (prymnesiophytes) (Fig. 2B).

The PCA based on the molecular analyses (Fig. 3A) showed no clear separation based on nutrient treatment, but there was a similar community change in all mesocosms with time. On Day 1, all treatments were to the left of the ordination and on Day 7 they were to the right. The taxa identified belonged to the chlorophytes (11 taxa), but also to other groups like Klebsormidiophyceae, Dinophyceae, Cercozoa and Ciliophora (Fig. 3B; Figs. S1 & S2 in the Supplement, www.int-res.com/articles/suppl/m489p093_supp.pdf).

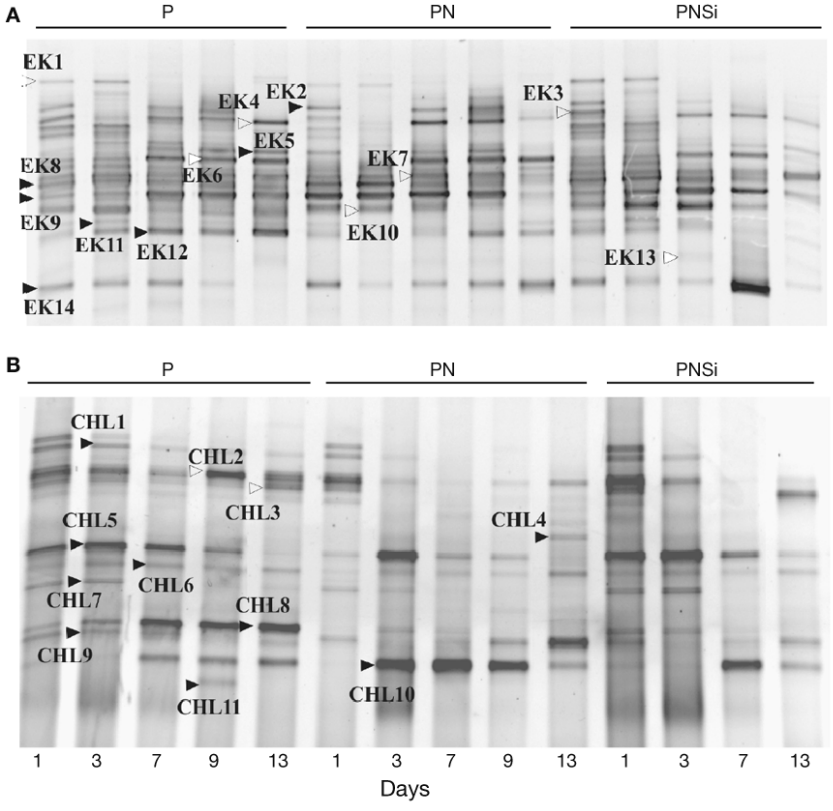

Fig. 3. DGGE fingerprint images showing different nutrient treatments. For identity results (phylogenetic reconstructions), see Supplementary material S1 and S2. (A) Image of the 18S rRNA gene analysis with universal eukaryotic primers, indicating the sequenced bands EK1-EK14. (B) Image of the 18S rRNA gene analysis with primers targeting chlorophytes, indicating the sequenced bands CHL1-CHL11. Abbreviations used in A: EK8, EK9, EK14 = Chlorophyta, EK2, EK 11, EK12, EK13 = Dinophyceae, EK3, EK7 = Cercozoa, EK4, EK10, EK1 = Ciliophora, EK6 = tentatively identified as heterotrophic stramenopiles, EK5 = uncertain affiliation. Abbreviations used in B: CHL8, CHL10 = Trebouxiophyceae, CHL4, CHL6 = Chlorophyceae, CHL1, CHL5, CHL7, CHL9, CHL11 = Prasinophyceae, CHL2 = Klebsormidiophyceae (Streptophyta), CHL3 = Cercozoa

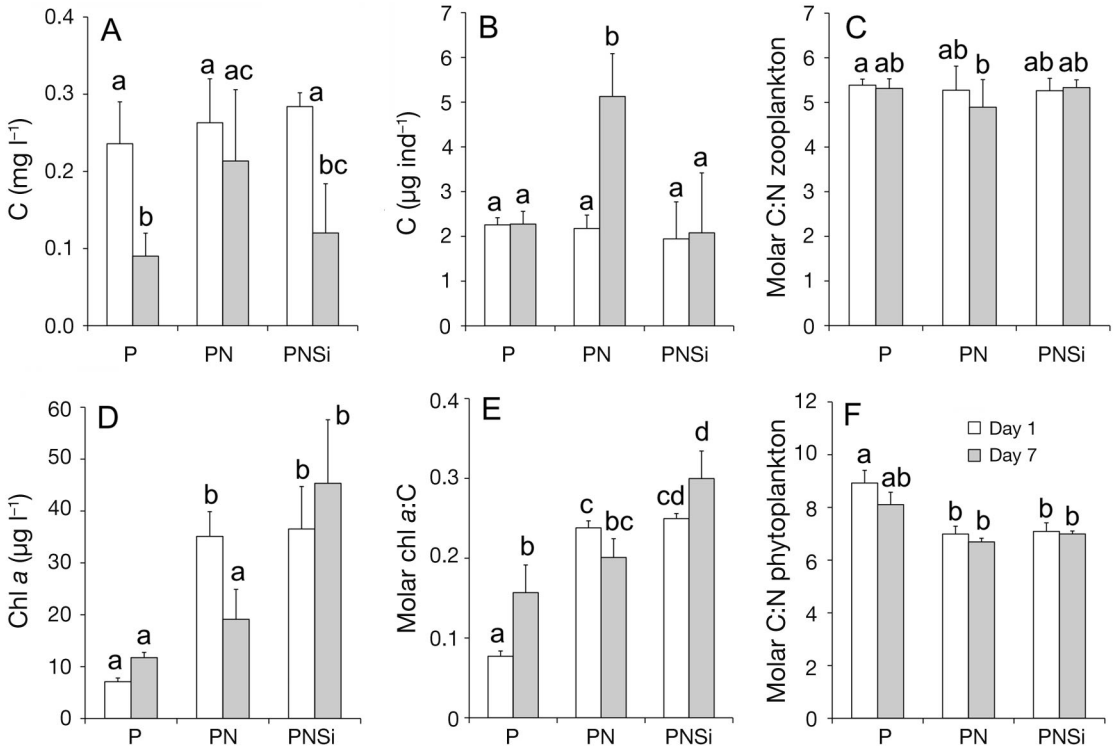

Fig. 4. Zoo- and phytoplankton biomass and stochiometry on Day 7 and Day 13 in Expt II. (A) Zooplankton biomass expressed as particulate C concentration. (B) Zooplankton carbon content per individual. (C) Molar C:N ratio in zooplankton. (D) Phytoplankton biomass expressed as chlorophyll *a* (chl *a*) concentration. (E) Molar chl *a*:C ratio in phytoplankton. (F) Molar C:N ratio in phytoplankton. Different letters above the bars indicate significant ($p < 0.05$) differences for the combined effects of treatment and time tested with post-hoc Tukey tests in repeated measures ANOVA analyses

Expt II, mesozooplankton

The initial mesozooplankton density in the mesocosms was 0.26 ± 0.04 mg C l^{-1} and 124 ± 23 ind. l^{-1} (mean ± SD, n = 9 mesocosms). The community was dominated by zooplankton of 500 to 650 µm in length, and consisted of the copepods *Acartia* spp. (relative abundance 67%), *Eurytemora affinis* (6%) and *Temora longicornis* (1%), and the cladoceran *Evadne nordmanni* (26%). In all 9 mesocosms, the zooplankton density decreased an equal amount in 6 d (repeated measures ANOVA, $p > 0.05$) to on average 58 ± 31 ind. l^{-1}. However, when the zooplankton community grazed on the PN-treated (Si-limited) phytoplankton, their carbon content per individual doubled while biomass remained the same (Fig. 4A,B). When the zooplankton grazed on P-treated (N-limited) or PNSi-treated phytoplankton, biomass decreased while the carbon content per individual remained the same (Fig 4A,B). The molar C:N ratio of the zooplankton (ca. 5.3) was not modified by any of the 3 phytoplankton communities (Fig. 4C). The community composition did not change significantly in the P- and PNSi-treatments; however, in the PN-treatment, the cladoceran became more abundant (43%) at the cost of all 3 copepod genera.

Expt II, phytoplankton

After 6 d of grazing, the phytoplankton biomass decreased in the PN-treated community, remained the same for the PNSi-treated community and increased in the P-treated community (Fig. 4D). The molar chl *a*:C ratio increased for the P-treated community, but not for the other 2 (Fig. 4E). The C:N ratio did not change for any of the phytoplankton communities, it remained high (N-limited) in the P-treated community and close to the Redfield ratio of 6.6 in the other 2 communities (Fig. 4F).

Expt II, community composition

The PCA results for the pigment and the genetic community data were very similar in Expt II

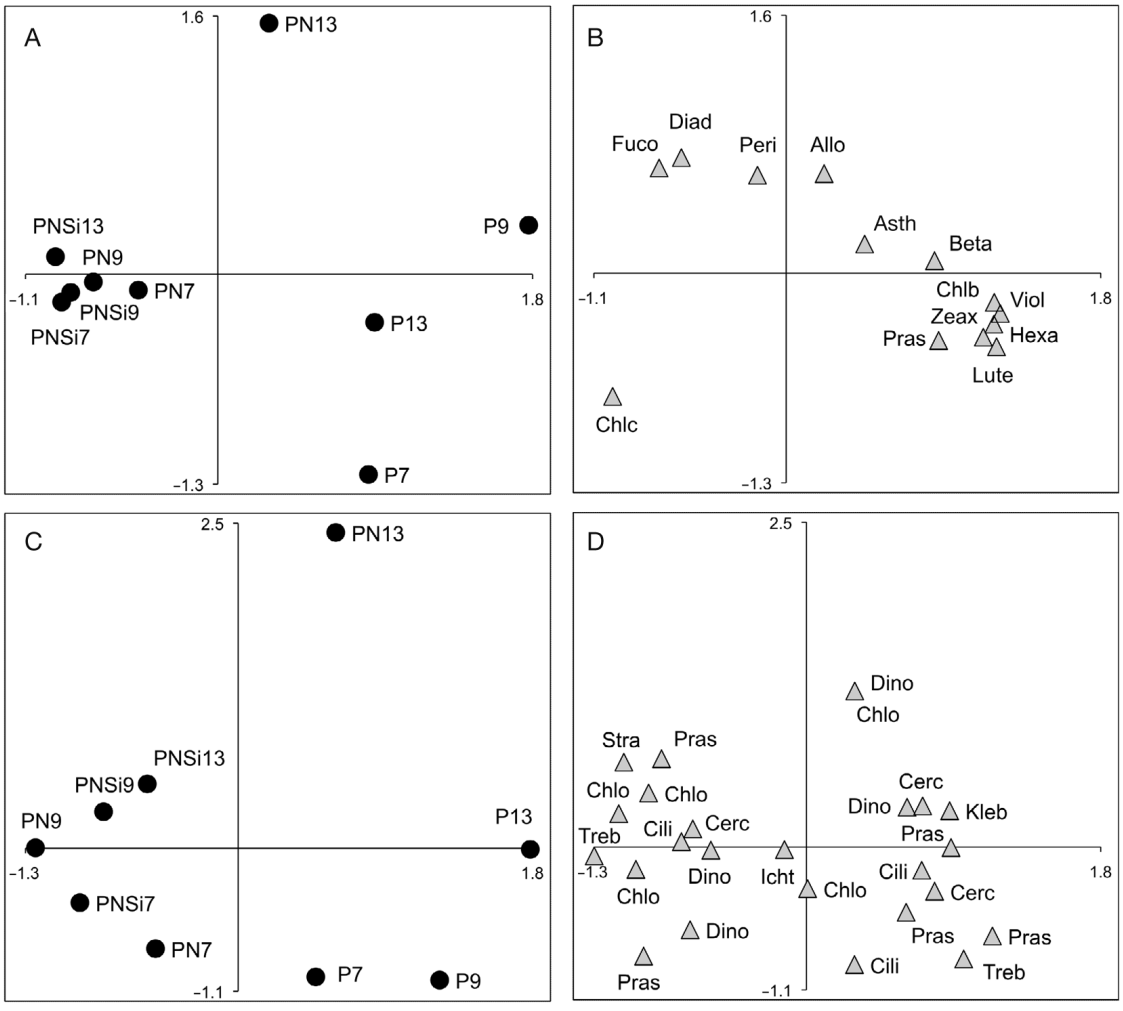

Fig. 5. PCA ordination plots showing changes in phytoplankton composition during Expt II. (A) Centroids for treatment/time combinations in the PCA analysis with the pigments (eigenvalues: Axis 1 = 0.60, Axis 2 = 0.17). (B) Scores for the pigments in the same PCA analysis as A. (C) Centroids for treatment/time combinations in the PCA analysis with the 18S rRNA phylotypes (eigenvalues: Axis 1 = 0.32, Axis 2 = 0.24). (D) Scores for the phylotypes in the same PCA analysis as C. Asth: asthaxanthin; other abbreviations as in Fig. 2

(Fig. 5A,C). The P- and PNSi-treated communities did not change much in the course of the experiment, which showed that the grazers had no effect on the community composition of the phytoplankton. The centroids of the P-treatment stayed to the right of the ordination, and those of the PNSi-treatment stayed to the left. In both ordinations, the centroid for the PN-treatment deviated from the others on Day 13 along Axis 2 and was found in the upper part of the ordination. This means that the grazers modified the community composition in the PN-treatment. This modification seemed to include increased abundances of dinoflagellates (peridinin) and cryptophytes (alloxanthin) and a chlorophyte species coded CHL4 (Fig. 5B,D; Fig. S2).

New DGGE optimization for chlorophytes

Our 18S rRNA gene analysis included a new DGGE optimization targeting chlorophytes. This optimization had high reproducibility as shown by the almost identical band patterns for the Day 1 samples in 3 different mesocosms (Fig. 3B). It yielded 15 DGGE bands of which 11 could be identified, and 9 of these were affiliated with chlorophytes (Fig. 3B; Fig. S2). The 18S rRNA gene analysis for eukaryotes with universal primers applied in this paper only resulted in 3 identified chlorophyte phylotypes, which in the present study were associated with Trebouxiophyceae, Prasinophyceae and Ulvophyceae. With the new DGGE optimization, the 15 bands retrieved pro-

vide a substantially higher resolution, especially within the prasinophytes (5 bands), which most likely represent DGGE band EK8 in the universal 18S rRNA gene analysis.

DISCUSSION

Si and nutritional quality

We found that the quality of phytoplankton as a food source for mesozooplankton in our experiment depended more on the phytoplankton Si:N stoichiometry than on their taxonomic composition. In the PN- and PNSi-treatments, the composition was basically the same on Day 7, despite the extremely low Si:N ratio in the PN-treatment of 0.03 (500 times lower than in the natural seawater). Thus, diatoms were able to grow well in the PN-treatment for 6 d while they depleted the available Si in the natural seawater. On Day 7, the DSi concentration in the PN-treatment was 0.3 µmol l^{-1}, which is below the critical level for diatom dominance of 2 µmol l^{-1} (Egge & Aksnes 1992), but diatoms still dominated the biomass. The zooplankton in the PN-treatment was actively grazing on the phytoplankton since they doubled their somatic C content in the PN-treatment while community composition shifted from copepods:cladocerans 73:27 to 57:43. This grazing activity is supported by the fact that the phytoplankton decreased in biomass and its community composition was modified between Day 7 and Day 13 in the PN-treatment, but not in the 2 other treatments. With the excess Si in the PNSi-treatment, ~60% of the zooplankton biomass died, with copepods and cladocerans affected equally. This may have been a density effect since the initial zooplankton density on Day 7 was high (124 ind. l^{-1}). However, in a similar mesocosm experiment, an *Acartia*-dominated community increased up to 243 ± 11 healthy ind. l^{-1} (n = 3 replicate mesocosms, P. Snoeijs unpubl.). The highest density we found in natural communities in the Baltic Sea was 54 ind. l^{-1} (Snoeijs & Häubner 2013). Irrespective of a possible density effect, it can be concluded that the zooplankton was not optimally fed by the large surplus of Si, despite high relative abundances and diversity of e.g. prasinophytes and heterotrophic flagellates and ciliates as shown by the genetic analysis. With this latter method, we found no general response patterns to the nutrient additions that could be attributed to algal classes. For example, different dinoflagellate and chlorophyte phylotypes could be positively or negatively affected by P-, NP- and PNSi-additions.

The diatoms growing in the PNSi-treatment used up 2.4 times more Si than those in the PN-treatment during 6 d. High Si:N ratios provide diatoms with possibilities to escape from grazing (heavy silicification, excretion of secondary metabolites), leading to the accumulation of algal biomass without transfer to higher trophic levels (Ban et al. 1997). Field studies have shown that the effects of diatoms on zooplankton can be either adverse (Miralto et al. 1999, Pierson et al. 2005) or favourable (Irigoien et al. 2002). Grazing selectivity in favour of diatoms has been demonstrated in copepods under natural conditions (Meyer-Harms et al. 1999, Irigoien et al. 2000) while other studies have reported a feeding preference for protozoans and flagellates (Nejstgaard et al. 2001, Peters et al. 2013). Our study shows that it is not the availability of diatoms per se that affects copepod somatic growth, but that also the Si:N stoichiometry of the diatoms determines their nutritional quality for the zooplankton.

In a mesocosm experiment in the western Baltic Sea, Sommer (2009) tested the feeding selectivity, growth and reproductive success of the copepod *Acartia tonsa* in mesocosms fertilized with 8 different Si:N ratios ranging from 0:1 to 1.75:1 while added N:P ratios were kept constant. In this experiment, very different phytoplankton community compositions developed with diatom biomass varying between 20 and 90%, depending on the nutrient ratios. *A. tonsa* strongly preferred feeding on motile prey (flagellates and ciliates) to feeding on diatoms. However, diatoms comprised a substantial part of the diet at the highest Si:N ratios. *A. tonsa* egg production and the final abundance of adults and copepodites showed no response to Si:N ratios while nauplii production slightly increased with Si:N ratios. Sommer (2009) concluded that even under high natural dominance of diatoms, there are always alternative food sources. This was also the case in our mesocosms, but still the copepods were not properly fed in the PNSi -treatment, which may indicate that secondary metabolites from the diatoms were involved when surplus Si was provided at high N and P concentrations. However, this was not proven in our study. Another possibility may be that species composition of diatoms and dinoflagellates differed between the PN- and PNSi-treatments, despite a very similar pigment composition. Exudates from dinoflagellates from the Baltic Sea were shown to cause incapacitation (erratic swimming motion, loss of motility and death) and no grazing (Sopanen et al. 2011).

Diekmann et al. (2009) found that Si-limited, senescent cultures of the diatom *Thalassiosira weissflogii* reduced secondary production in terms of both egg production rate and copepodite development rate in *Acartia tonsa*, compared to when the copepods were fed diatoms maintained in nutrient replete conditions. They suggested that reductions in copepod production and standing stocks often observed at the end of diatom blooms may not only be due to reductions in food quantity (and losses due to predators), but also that remaining diatoms may be an exceptionally poor quality food source for grazers. This seems opposite to our Si-limited diatom-dominated communities, which had a positive effect on zooplankton somatic growth. However, in both the study by Diekmann et al. (2009) and in our mesocosms, diatom-produced toxic metabolites may have interfered with the quality of diatoms as food for the mesozooplankton in different ways. Also, it is almost impossible to compare the effect of single-culture studies (*T. weissflogii*) to whole phytoplankton communities (our mesocosms).

N-limitation and nutritional quality

We found that a phytoplankton community with stoichiometry close to the C:N Redfield ratio of 6.6 (PN-treatment) provided better nutritional quality for the zooplankton than the natural C:N of the initial phytoplankton of almost 9 (P-treatment). The latter is a high C:N ratio even for the Baltic Sea phytoplankton, but not unusual (Walve & Larsson 2010). It can be argued that the biomass was higher in the PN- and PNSi-treatments than in the P-treatment (C concentrations ~50% and chl *a* concentrations ~450% higher) and that competition for prey would, therefore, be greater in the P-treatment. However, there was no shortage of food in the P-treatment during the 6 d grazing experiment since the phytoplankton biomass and also the chl *a*:C ratio increased. It is more likely that the zooplankton was not optimally fed by the strongly N-limited phytoplankton despite high relative abundances and diversity of green algae as shown by the genetic analysis. Evidence of this was not observed in the C:N ratio of the zooplankton, which stayed around 5 throughout the experiment, which is normal for *Acartia*, *Eurytemora* and *Evadne* in the Baltic Sea. Walve & Larsson (1999) reported C:N ratios of 4.5 to 4.8 for *Acartia* and *Eurytemora* and 5.1 to 5.8 for *Evadne*. On the other hand, these were the zooplankton individuals that survived in the P-treatment, 60% of the biomass was lost. Grazers often possess physiological solutions to the problem of excess C in their diet by not assimilating C across the gut wall or by disposing assimilated C through respiration or extracellular release of organic C-compounds (Sterner & Hessen 1994). Nevertheless, Van Nieuwerburgh et al. (2004) showed that strong N-limitation in phytoplankton cells with a C:N ratio of 8 to 13 can be transferred to copepod grazers.

The Baltic Sea is notorious for its cyanobacterial blooms in summer. These blooms are dominated by filamentous *Nodularia*, *Aphanizomenon* and *Anabaena* species, and are a natural feature in the Baltic Sea during N-limitation in summer (Suikkanen et al. 2007, Jaanus et al. 2011). Increased growth of cyanobacteria could be expected in our P-treatment, which had much stronger N-limitation after 6 d than the initial seawater. However, the pigment composition of the initial phytoplankton community contained no echinenone, which is a marker pigment for *Nodularia* (Sylvander et al. 2013). No echinenone was detected in the course of the experiment either. Zeaxanthin, which occurs both in cyanobacteria and some green algae, remained high in the P-treatment but decreased in the other 2 treatments, which may indicate a higher biomass of picocyanobacteria in the P-treatment. However, since this pigment is not specific for cyanobacteria, there is no proof of this.

Acknowledgements. Financial support from The Swedish Research Council for Environment, Agricultural Science and Spatial Planning (to B.D.), the Knut and Alice Wallenberg Foundation and the Norsk Hydro Research Foundation (to P.S.), and the Ebba and Sven Schwarz' Foundation (to L.V.N.) is acknowledged. We are grateful to Rostock University (Germany) for providing research facilities at the Zingst Biological Station. We thank M. Andersson, M. Paes de Barros, H. Baudler, P. Feuerpfeil, J. Johansson, D. Larson, M. McCausland, V. Reiff, H. Schubert for advice, assistance and good company during the mesocosm experiment. We are grateful to 3 anonymous reviewers for excellent comments to the manuscript.

LITERATURE CITED

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 56: 1919–1925

Andersson M, Van Nieuwerburgh L, Snoeijs P (2003) Pigment transfer from phytoplankton to zooplankton with emphasis on astaxanthin production in the Baltic Sea

food web. Mar Ecol Prog Ser 254:213–224
Ban S, Burns C, Castel J, Chaudron Y and others (1997) The paradox of diatom-copepod interactions. Mar Ecol Prog Ser 157:287–293
Brzezinski MA (1985) The Si-C-N ratio of marine diatoms—interspecific variability and the effect of some environmental variables. J Phycol 21:347–357
Conley DJ, Schelske CL, Stoermer EF (1993) Modification of the biogeochemical cycle of silica with eutrophication. Mar Ecol Prog Ser 101:179–192
Conley DJ, Humborg C, Smedberg E, Rahm L and others (2008) Past, present and future state of the biogeochemical Si cycle in the Baltic Sea. J Mar Syst 73:338–346
Diekmann ABS, Peck MA, Holste L, St John MA, Campbell RW (2009) Variation in diatom biochemical composition during a simulated bloom and its effect on copepod production. J Plankton Res 31:1391–1405
Díez B, Pedrós-Alió C, Marsh TL, Massana R (2001) Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl Environ Microbiol 67:2942–2951
Egge JK, Aksnes DL (1992) Silicate as regulating nutrient in phytoplankton competition. Mar Ecol Prog Ser 83: 281–289
Estrada M, Henriksen P, Gasol JM, Casamayor EO, Pedrós-Alió C (2004) Diversity of planktonic photoautotrophic microorganisms along a salinity gradient as depicted by microscopy flow cytometry pigment analysis and DNA-based Methods. FEMS Microbiol Ecol 49:281–293
Granéli E, Wallström K, Larssson U, Granéli W, Elmgren R (1990) Nutrient limitation of primary production in the Baltic Sea area. Ambio 19:142–151
Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. Can J Microbiol 8:229–239
HELCOM (Helsinki Commision) (2009) Eutrophication in the Baltic Sea—an integrated thematic assessment of the effects of nutrient enrichment in the Baltic Sea region. HELCOM Baltic Sea Environ Proc 115B:1–148
Irigoien X, Head RN, Harris RP, Cummings D, Harbour D (2000) Feeding selectivity and egg production of *Calanus helgolandicus* in the English channel. Limnol Oceanogr 45:44–54
Irigoien X, Harris RP, Verheye HM, Joly P and others (2002) Copepod hatching success in marine ecosystems with high diatom concentrations. Nature 419:387–389
Jaanus A, Andersson A, Olenina I, Toming K, Kaljurand K (2011) Changes in phytoplankton communities along a north–south gradient in the Baltic Sea. Boreal Environ Res 16(Suppl. A):191–208
Jeffrey SW, Vesk M (1997) Introduction to marine phytoplankton and their pigment signaturas. In: Jeffrey SW Mantoura RFC Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. Unesco, Paris, p 37–84
Kiørboe T (1997) Population regulation and role of mesozooplankton in shaping marine pelagic food webs. Hydrobiologia 363:13–27
Klais R, Tamminen T, Kremp A, Spilling K, Olli K (2011) Decadal-scale changes of dinoflagellates and diatoms in the anomalous Baltic Sea spring bloom. PLoS ONE 6: e21567
Lim EL, Amaral LA, Caron DA, DeLong EF (1993) Application of ribosomal RNA-based probes for observing marine nanoplanktonic protists. Appl Environ Microbiol 59: 1647–1655
Mackey MD, Mackey JD, Higgins HW, Wright SW (1996) CHEMTAX—a program for estimating class abundances from chemical markers: application to HPLC measurements of phytoplankton. Mar Ecol Prog Ser 144:265–283
Massana R, Guillou L, Díez B, Pedrós-Alió C (2002) Unveiling the organisms behind novel eukaryotic ribosomal DNA sequences from the ocean. Appl Environ Microbiol 68:4554–4558
Meyer-Harms B, Irigoien X, Head R, Harris R (1999) Selective feeding on natural phytoplankton by *Calanus finmarchicus* before, during, and after the 1997 spring bloom in the Norwegian Sea. Limnol Oceanogr 44:154–165
Miralto A, Barone G, Romano G, Poulet SA and others (1999) The insidious effect of diatoms on copepod reproduction. Nature 402:173–176
Moon-van der Staay SY, DeWachier R, Vaulot D (2001) Oceanic 18S rDNA sequences picoplankton reveal unsuspected eukaryotic diversity. Nature 409:607–610
Nejstgaard JC, Hansen Hygum B, Naustvoll LJ, Båmstedt U (2001) Zooplankton growth, diet and reproductive success compared in simultaneous diatom- and flagellate-microzooplankton-dominated-plankton blooms. Mar Ecol Prog Ser 221:77–91
Olli K, Klais R, Tamminen T, Ptacnik R, Andersen T (2011) Long-term changes in the Baltic Sea phytoplankton community. Boreal Environ Res 16(Suppl. A):3–14
Olsen Y, Agustí S, Andersen T, Duarte CM and others (2006) A comparative study of responses in planktonic food web structure and function in contrasting European coastal waters exposed to experimental nutrient addition. Limnol Oceanogr 51:488–503
Paerl HW, Valdes LM, Pinckney JL, Piehler MF, Dyble J, Moisander PH (2003) Phytoplankton photopigments as indicators of estuarine and coastal eutrophication. Bioscience 53:953–964
Peters J, Dutz J, Hagen W (2013) Trophodynamics and life-cycle strategies of the copepods *Temora longicornis* and *Acartia longiremis* in the central Baltic Sea. J Plankton Res 35:595–609
Pierson JJ, Halsband-Lenk C, Leising AW (2005) Reproductive success of *Calanus pacificus* during diatom blooms in Dabob Bay, Washington. Prog Oceanogr 67:314–331
Pinto E, Van Nieuwerburgh L, Barros MP, Pedersén M, Colepicolo P, Snoeijs P (2003) Density-dependent patterns of thiamine and pigment production in *Nitzschia microcephala.* Phytochemistry 63:155–163
Redfield AC, Ketchum BH, Richards FA (1963) The influence of organisms on the composition of sea-water. In: Hill MN (ed) The sea. Wiley-Liss, New York, NY p 26–77
Schauer M, Massana R, Pedrós-Alió C (2000) Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. FEMS Microbiol Ecol 33:51–59
Simon N, Campbell L, Ornolfsdottir E, Groben R, Guillou L, Lange M, Medlin LK (2000) Oligonucleotide probes for the identification of 3 algal groups by dot blot and fluorescent whole-cell hybridization. J Eukaryot Microbiol 47:76–84
Snoeijs P, Häubner N (2013) Astaxanthin dynamics in Baltic Sea mesozooplankton communities. J Sea Res. doi: 10.1016/j.seares.2013.04.015

Sogin ML, Gunderson JH (1987) Structural diversity of eukaryotic small subunit ribosomal RNAs. Ann NY Acad Sci 503:125–139

Sommer U (1994) Are marine diatoms favourised by high Si: N ratios? Mar Ecol Prog Ser 115:309–315

Sommer U (2009) Copepod growth and diatoms: insensitivity of *Acartia tonsa* to the composition of semi-natural plankton mixtures manipulated by silicon:nitrogen ratios in mesocosms. Oecologia 159:207–215

Sopanen S, Setälä O, Piiparinen J, Erler K, Kremp A (2011) The toxic dinoflagellate *Alexandrium ostenfeldii* promotes incapacitation of the calanoid copepods *Eurytemora affinis* and *Acartia bifilosa* from the northern Baltic Sea. J Plankton Res 33:1564–1573

Sterner RW, Hessen DO (1994) Algal nutrient limitation and the nutrition of aquatic herbivores. Annu Rev Ecol Syst 25:1–29

Suikkanen S, Laamanen M, Huttunen M (2007) Long-term changes in summer phytoplankton communities of the open northern Baltic Sea. Estuar Coast Shelf Sci 71: 580–592

Sylvander P, Häubner N, Snoeijs P (2013) The thiamine content of phytoplankton cells is affected by abiotic stress and growth rate. Microb Ecol 65:566–577

ter Braak CJF, Smilauer P (2002) CANOCO reference manual and CanoDraw for Windows user's guide: software for canonical community ordination (version 4.5). Microcomputer Power, Ithaca, NY

Tillett D, Neilan BA (2000) Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria. J Phycol 36:251–258

Van Nieuwerburgh L, Wänstrand I, Snoeijs P (2004) Growth and C:N:P ratios in copepods grazing on N- or Si-limited phytoplankton blooms. Hydrobiologia 514:57–72

Walve J, Larsson U (1999) Carbon, nitrogen and phosphorus stoichiometry of crustacean zooplankton in the Baltic Sea: implications for nutrient recycling. J Plankton Res 21:2309–2321

Walve J, Larsson U (2010) Seasonal changes in Baltic Sea seston stoichiometry: the influence of diazotrophic cyanobacteria. Mar Ecol Prog Ser 407:13–25

Wasmund N, Uhlig S (2003) Phytoplankton trends in the Baltic Sea. ICES J Mar Sci 60:177–186

Wright SW, Jeffrey SW (1997) High-resolution HPLC system for chlorophylls and carotenoids of marine phytoplankton. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. Unesco, Paris, p 327–341

Wright SW, Jeffrey SW, Mantoura RFC (1997) Evaluation of methods and solvents for pigment extraction. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography: guidelines to modern methods. Unesco, Paris, p 261–282

Yan QY, Yu YH, Feng WS, Deng WN, Song SH (2007) Genetic diversity of plankton community as depicted by PCR-DGGE fingerprinting and its relation to morphological composition and environmental factors in Lake Donghu. Microb Ecol 54:290–297

Zhu F, Massana R, Not F, Marie D, Vaulot D (2005) Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. FEMS Microbiol Ecol 52: 79–92

Editorial responsibility: Antonio Bode, A Coruña, Spain

Submitted: October 29, 2012; Accepted: June 7, 2013
Proofs received from author(s): August 12, 2013