Pigment transfer from phytoplankton to zooplankton with emphasis on astaxanthin production in the Baltic Sea food web

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ABSTRACT: The carotenoid astaxanthin is a powerful antioxidant and a compound of vital importance for many marine organisms such as crustaceans and salmonids. Although astaxanthin deficiency may have serious consequences for ecosystem functioning, its origin and transfer in the food web have been little studied. Astaxanthin is produced by planktonic crustaceans, but these cannot synthesise carotenoid pigments de novo; they rely on the availability of astaxanthin precursors through the consumption of phytoplankton algae. We performed 4 laboratory experiments to test how the production of astaxanthin in wild pelagic copepod communities (mainly consisting of *Acartia*) is affected when different phytoplankton communities (unfertilised, fertilised with N and P, or fertilised with N, P and Si) are supplied as prey. We show that phytoplankton community composition and biomass have profound effects on the production of astaxanthin in calanoid copepods. When they were grazing on a diverse phytoplankton community with high biomass dominated by chlorophytes, dinoflagellates and diatoms with thin silica frustules, astaxanthin production in the copepods was highest. It was lower when the copepods were fed with low phytoplankton biomass or high biomass dominated by large heavily silicified diatoms; these diatoms were not consumed and grazing was mainly on prymnesiophytes. When the astaxanthin production was high, maximum astaxanthin content per copepod individual of about twice the initial level was reached during sunrise. These results suggest increased feeding activity and astaxanthin production during the night and utilisation of astaxanthin for photoprotection and other antioxidant activities during the day.

KEY WORDS: Astaxanthin · Carotenoids · Food web · Phytoplankton · Zooplankton · Copepods · Grazing · Baltic Sea

INTRODUCTION

Large-scale environmental changes have occurred in the brackish Baltic Sea during the last 50 yr, and some of these changes have been caused by eutrophication (Elmgren 1989, Jansson & Dahlberg 1999). Shifts in phytoplankton community composition are a universal consequence of increased availability of nitrogen and/or phosphorus (Snoeijs 1999). This, in turn, may affect the whole food web since the primary producers provide vital nutrients for higher trophic levels. For example, the M74 syndrome, a reproductional disturbance in the Baltic populations of the Atlantic salmon *Salmo salar* L., is generally considered to be related to large-scale environmental changes (Lundström et al. 1998, Bengtsson et al. 1999). This serious threat to wild Baltic salmon stocks has been shown to be associated with low thiamine and astaxanthin levels in fish fry (Amcoff et. al 1998, Pettersson & Lignell 1999) and alterations in fatty acid composition in eggs (Pickova et al. 1999).

Studies from the Black and Baltic Seas have shown that eutrophication increases primary production, and diatoms are replaced by other phytoplankton groups such as dinoflagellates and prymnesiophytes (Leppäkoski & Mihnea 1996). However, when silicate is not limiting, diatoms are usually the best competitors. This has been shown in numerous laboratory and mesocosm

experiments (e.g. Sommer 1994, Jacobsen et al. 1995). Although it is well known that copepods do not feed exclusively on diatoms (Paffenhöfer 2002), diatoms are generally thought to serve as a high-quality food source for higher trophic levels, such as copepods (Runge 1988, Legendre 1990, Koski et al. 1998). However, recently this function of diatoms in the food web has also been questioned and is a matter of debate, as it challenges the classical view of marine food web energy flow from diatoms to fish by means of copepods (Kleppel 1993, Jónasdóttir et al. 1998). Based on both laboratory and *in situ* studies, it has been proposed that many diatom species, especially at bloom concentrations, are able to inhibit the production and viability of pelagic copepods' eggs (Ban et al. 1997, Miralto et al. 1999, Paffenhöfer 2002). However, in a recent large data compilation, no negative relationships could be detected between copepod egg hatching success and either diatom biomass or dominance in the microplankton in any of 12 globally distributed areas where diatoms dominate the phytoplankton community (Irigoien et al. 2002). Further phytoplankton characteristics that have been shown to affect the rates of development, somatic growth and reproduction of copepods include cell size, cell morphology, toxicity, mineral composition and content of some biochemical components, such as amino acids, polyunsaturated fatty acids, sugars and vitamins (Koski et al. 1998 and references therein). However, little is known about the transfer of carotenoids from phytoplankton to pelagic copepods.

In the present study we investigate whether the production of the important antioxidant astaxanthin (Edge et al. 1997) in copepods depends on their microalgal diet. Pigments can be used to trace the algal diet of zooplankton because pigment composition typically varies between phytoplankton groups, e.g. alloxanthin is typical of cryptophytes, peridinin of dinoflagellates, prasinoxanthin of prasinophytes, 19′-hexanoyloxy-fucoxanthin of prymnesiophytes, chlorophyll *b* (chl *b*) of chlorophytes, prasinophytes and euglenophytes, fucoxanthin of diatoms, prymnesiophytes, chrysophytes and raphidophytes, etc. (Jeffrey & Vesk 1997). β-carotene obtained from phytoplankton is considered to be the main precursor for astaxanthin synthesis in zooplankton via other carotenoids such as zeaxanthin and lutein (Katayama et al. 1973, Kleppel et al. 1985). Although β-carotene basically occurs in all microalgae, quantitative differences occur between phytoplankton

groups, while zeaxanthin and lutein only occur in some groups, notably cyanobacteria and chlorophytes (Jeffrey & Vesk 1997). We performed 4 laboratory experiments to test if and how the production of astaxanthin in wild copepod communities (mainly consisting of *Acartia*) is affected when different phytoplankton communities (unfertilised, fertilised with N and P, or fertilised with N, P and Si) are supplied as food.

MATERIALS AND METHODS

The experiments were carried out at the Zingst field station, Germany, in the southern Baltic Sea proper (54° 25′ N, 12° 40′ E). Phytoplankton communities were obtained from 1600 l mesocosms where different phytoplankton blooms were created by manipulations of natural seawater (NSW) of 8.5 psu with nutrients (Table 1). For further description of the mesocosms, see Barros et al. (2003). In the morning of each sampling day, 25 l of water were sampled (after careful mixing of the water in the mesocosms) from each of 3 replicate mesocosms, and filtered through a 100 µm mesh plankton net. Thus, the final phytoplankton stock consisted of 75 l seawater with organisms <100 µm in a large plastic container, which was stored in the laboratory until the start of the experiment on the afternoon of the same day. For the NSW treatment, the water was sampled directly from the sea, where the copepod stock was also collected.

For each experiment, new calanoid copepods were collected with a 200 µm mesh size plankton net at 0 to

Table 1. Summary of the 4 different treatments of the phytoplankton communities prior to the grazing experiments. The NSW treatment consisted of natural seawater sampled directly from the Baltic Sea. For the other 3 treatments phytoplankton blooms were created in 1600 l mesocosms by nutrient additions. Each day, the mesocosms also received trace metals, chelators and vitamins in 0.5% of f/2 medium concentrations (Guillard & Ryther 1962) but excluding thiamine

Treatment	Daily nutrient additions to the mesocosms	Number of days of mesocosm cultivation	Sampling day
NSW	None	0	1 September 2002
NP	4 µM $NaNO_3$ 0.25 µM NaH_2PO_4 28 µM $NaHCO_3$	11	11 July 2001
NPSi1	4 µM $NaNO_3$ 0.25 µM NaH_2PO_4 12 µM Na_2SiO_3 28 µM $NaHCO_3$	9	9 July 2001
NPSi2	4 µM $NaNO_3$ 0.25 µM NaH_2PO_4 12 µM Na_2SiO_3 28 µM $NaHCO_3$	11	12 September 2001

10 m depth, about 1.5 km offshore. Sampling took place in the morning of each experimental day and the copepods were stored in a 10 l container in NSW for transport to the laboratory. Within 0.5 h of sampling the copepod sample was diluted to ~50 l with filtered NSW and cleansed of macroalgae and dead (floating) zooplankton. The final copepod stock was stored in the laboratory until the start of the experiment. Thus, the copepods were starved for ~6 h prior to each experiment. A quick estimate of the copepod density in the stock was made by counting the number of individuals in triplicate 100 ml well-mixed samples. For each experiment, 3 samples from the copepod stock were fixed with Lugol's solution for later identification and exact density determination.

All experiments were started between 16:00 and 17:00 h. Altogether, thirty-nine 2 l polypropylene jars (height 18 cm, diameter 14 cm) were filled with 1.5 l of phytoplankton stock, then placed on a table in the laboratory exposed to natural daylight (but not to direct sunlight to avoid aggregations of copepods and flagellates) through an open door. Room temperature was 20 to 25°C. A fixed volume of the copepod stock (based on the fast estimates of copepod density) was poured through plexiglas insets (height 16 cm, diameter 13 cm) with 200 µm mesh size plankton net bottoms to obtain an approximate density of 50 copepod individuals l^{-1} (75 inset^{-1}). One inset with copepods was placed in each jar. Thus, the phytoplankton (<100 µm) that was already in the seawater in the jars and the copepods (>200 µm) on the net of the inset were mixed. The triplicate zero sampling ($t = 0$ h) took place before copepods were added to the other 36 jars. These jars were randomly sampled in triplicate after $t = 1, 2, 3, 4,$ 5, 7, 9, 12, 15, 18, 21 and 24 h of copepod grazing. The copepod samples (including the $t = 0$ samples) consisted of all copepods from 1 jar, and were obtained by taking out the inset and rinsing the net with filtered seawater. Phytoplankton samples (110 to 150 ml) were taken from each jar after careful mixing. The copepod samples were filtered on Whatman™ GF/C filters, and the phytoplankton samples on Whatman™ GF/F filters. During filtering it was observed that all or the vast majority of the copepods were alive at this point, in all experiments. The filters were wrapped in aluminium foil, immediately frozen in liquid nitrogen and within 2 wk transported to Uppsala, where they were stored at −86°C in a freezer until pigment analyses were carried out (within 4 mo).

Chlorophylls and carotenoids were extracted from the filters according to Wright et al. (1997). The phytoplankton filters were sonicated for 1 min in cold methanol on ice (Vibra Cell™, pulse 0.9 s, amplitude 92) and centrifuged for 4 min at 4°C and $6000 \times g$, after which the pigments were immediately analysed. The copepod filters were sonicated for 1 min in cold acetone on ice (Vibra Cell™, pulse 0.9 s, amplitude 92), left overnight in the dark at 4°C (Buffan-Dubau & Carman 2000), centrifuged for 4 min at 4°C and $6000 \times g$, after which the pigments were immediately analysed.

For pigment separation the reversed-phase HPLC method of Wright & Jeffrey (1997) was used with a slightly modified solvent system program. A volume of 100 to 150 µl was injected into the HPLC system using an autoinjector (Spark Holland™, Promis II). Two different reversed-phase C-18 columns were used: a Spherisorb 5ODS, 250×4.60 mm, 5 µm particle size, Phenomenex™ for the phytoplankton samples and a Reprosil-Pur C18-Aq column, 250×4 mm, 5 µm particle size, Dr. A. Maisch™ for the copepod samples. The first column separated algal pigments better (e.g. it gave an almost complete separation of lutein and zeaxanthin), while the second column detected astaxanthin. 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). Pigment calibration was achieved using a 5-point calibration curve (with dilutions made in triplicate = 15 injections) with the following pigment standards: canthaxanthin and zeaxanthin from Roth™, astaxanthin, β,β-carotene, β,ε-carotene, chlorophyll *a* (chl *a*), chl *b*, lutein and lycopene from Sigma™, alloxanthin, antheraxanthin, 19′-butanoyloxy-fucoxanthin, chlorophyll *c* (chl *c*), diadinoxanthin, diatoxanthin, echinenone, fucoxanthin, 19′-hexanoyloxy-fucoxanthin, peridinin, prasinoxanthin and violaxanthin from DHI Water & Environment™, Denmark. Mono- and diester forms of astaxanthin were identified with the help of cultivated *Haematococcus pluvialis* cysts in which esterified forms of astaxanthin dominate the pigment composition, and they were quantified using the calibrated equation for free astaxanthin.

Microscopic observations established that less than 2 % of the cells in our 4 phytoplankton stocks consisted of small zooplankton (some ciliates) and heterotrophic flagellates, and no larger zooplankton organisms occurred in these stocks. Therefore we assumed in our calculations that the pigments in the phytoplankton samples included negligible amounts of zooplankton pigments. We also observed that some of the calanoid copepods carried epizoic microalgae on their body surfaces, predominantly chlorophytes, but also some diatoms. This is a problem when quantifying the transfer of carotenoids from phytoplankton to copepods. Another problem was that, unavoidably, some microalgae from the phytoplankton stock became trapped on the 200 µm net when sampling the copepods, and thus became a part of the copepod samples. The third problem was that the copepods still may have had some algal pigments in their guts, even after ~6 h of starvation. To

solve these problems, we designed a net transfer ratio (TR_{net}, Fig. 1) to enable us to compare the transfer of carotenoids from the different phytoplankton communities to the copepods. The TR_{net} was defined as $TR_{net} = TR_{t=z} - TR_{t=0}$, where $TR = ([\mu mol\ l^{-1}$ of carotenoid in the copepod sample]/[$\mu mol\ l^{-1}$ of chl a in the copepod sample])/([$\mu mol\ l^{-1}$ of carotenoid in the phytoplankton sample]/[$\mu mol\ l^{-1}$ of chl a in the phytoplankton sample]). For algal carotenoids used by the copepods to synthesise body pigments (e.g. β-carotene, zeaxanthin, lutein), a positive TR_{net} expresses the degree of enrichment of a specific carotenoid by the copepods and a negative TR_{net} the degree of loss of this carotenoid from the copepods. For algal carotenoids not used by the copepods to synthesise body pigments (e.g. 19′-hexanoyloxy-fucoxanthin, peridinin, fucoxanthin, diadinoxanthin), a positive TR_{net} expresses the degree of selective grazing by the copepods on certain groups of microalgae and a negative TR_{net} expresses selection against certain groups of microalgae.
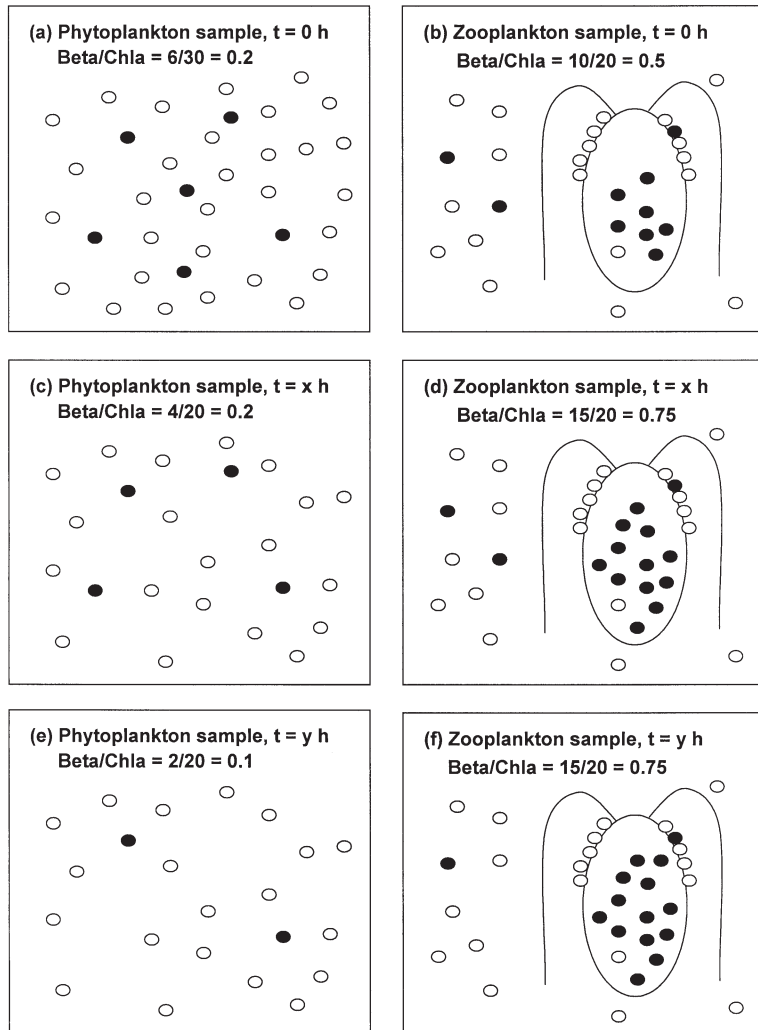
To summarise the variation in pigment composition among the samples, principal component analysis (PCA), implemented with the programme CANOCO (ter Braak & Šmilauer 1998), was applied to relative abundances of the pigments on a molar basis. To describe similarity in pigment composition between 2 samples, the Sørensen similarity index for quantitative samples was calculated, based on the same relative abundances: $S = (2\ M/[M_1 + M_2]) \times 100\%$, where M is the sum of the smaller values of the species common to both samples, M_1 is the sum of all values in the first sample and M_2 is the sum of all values in the second sample. (Mueller-Dombois & Ellenberg 1974). In our case, this measure of similarity varies from 0% for 2 samples with no carotenoids in common to 100% for 2 samples with identical carotenoid composition. Linear regression analyses were carried out with the programme MINITAB™ Version 13.1.

Fig. 1. Explanation of the transfer ratio (TR_{net}) used for comparisons of the transfer of carotenoids from the different phytoplankton communities to the copepods, using different scenarios. Open circles represent 1 molecule of chlorophyll a (chl a) and black dots represent 1 molecule of β-carotene (beta). (a) and (b) illustrate the $t = 0$ sampling. Phytoplankton sample contains molecules of beta and chl a in a certain ratio (here 0.2) and the copepod sample contains beta and chl a in a certain ratio (here 0.5). In the copepod sample the ratio depends on the concentrations of these pigments in and on the copepod body and in the fraction of the phytoplankton sample trapped on the 200 µm plankton net. (c) and (d) illustrate sampling after × h of grazing where the ratio in the phytoplankton has stayed the same (no selective grazing), and the copepods have enriched beta in their bodies while chl a has been excreted or destroyed. In this case $TR_{net} = TR_x - TR_0 = (0.75/0.2) - (0.5/0.2) = 1.25$. (e) and (f) illustrate sampling after y h of grazing where the ratio in the phytoplankton has changed due to selective grazing on a specific algal group, and the copepods have enriched beta in their bodies while chl a has been excreted or destroyed. In this case $TR_{net} = TR_x - TR_0 = (0.75/0.1) - (0.5/0.2) = 5.0$

RESULTS

Altogether, 25 pigments were identified in the phytoplankton samples and 25 in the copepod samples. Of these pigments, 11 occurred in both sample types (with zeaxanthin/lutein considered as 1). The sample scores of the PCA analysis showed that the phytoplankton pigment compositions of the 2 NPSi treatments

were similar (Fig. 2a). Also the phytoplankton pigment compositions of the NSW and NP treatments were similar to each other, but quite unlike those of the NPSi treatments (Fig. 2a). The 2 sample pairs NSW/NP and NPSi1/NPSi2 were separated along the first ordination axis (Axis 1). This axis explained most of the variation in the data set; the eigenvalues of the first 4 axes were 0.70, 0.24, 0.02 and 0.01, respectively. The Sørensen similarity index showed that the carotenoids in the 2 NPSi treatments were most similar (S = 90%), but the NSW and NP treatments were also rather similar (S = 76%). Similarity was lower between the NSW and NP treatments on one side and the 2 NPSi treatments on the other (S = 42 to 51%). The copepod sample scores were separated from those of the phytoplankton along Axis 2 for the NSW and NP treatments and mainly along Axis 1 for the 2 NPSi treatments. The pigment scores of the same ordination (Fig. 2b) showed that chl c, fucoxanthin and diadinoxanthin were most associated with the phytoplankton samples of the NPSi treatments, whereas most other algal pigments were associated with the phytoplankton samples of the NSW and NP treatments. This indicates a larger pigment diversity in the NSW and NP treatments and dominance of diatoms in the NPSi treatments. Typical zooplankton pigments were found in the upper-left quadrant of the ordination, along with β-carotene and pheophorbide a, 2 pigments that occurred in both phytoplankton and copepod samples, but with higher relative abundances in the copepods.

The biomass in the phytoplankton samples, expressed as chl a concentration, was highest in the NPSi1 treatment (59 nmol chl a l^{-1}) and lowest in the NSW treatment, which reflected the natural Baltic Sea conditions in September 2002 (7 nmol chl a l^{-1}) (Table 2). Significant (p < 0.05) negative linear regression coefficients between incubation time in h and pmol chl a l^{-1} were found for 3 of the 4 experiments (Ref: –47; NP: –340; NPSi2: –220) (Table 3). This may be considered a rough measure of grazing, but is in fact a complex combination of grazing and algal growth factors. The strongest decreasing trend in biomass (8.2 nmol chl a l^{-1} in 24 h) was observed in the NP treatment.

The major carotenoids in the phytoplankton of the NPSi treatments were fucoxanthin and diadinoxanthin, 66 to 71% and 11 to 17% of all carotenoids, respectively (Table 2). Together with high chl c concentrations (Table 2), these carotenoids indicate that the biomass was completely dominated by diatoms in the NPSi treatments. This was
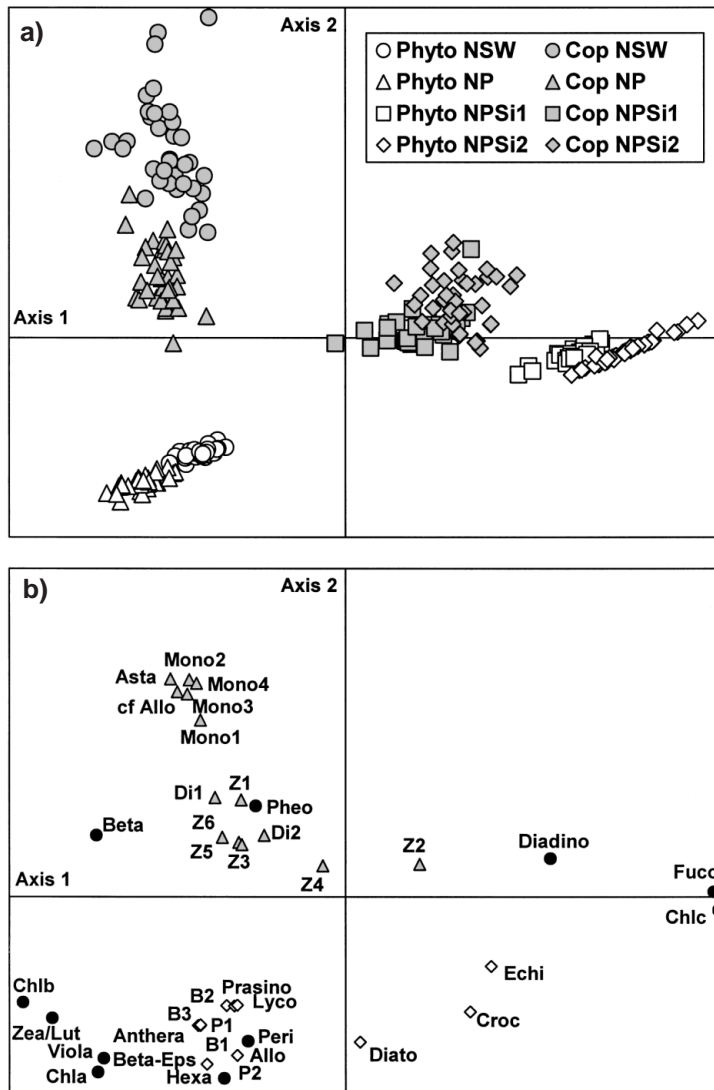

Fig. 2. Ordination plot of the PCA analysis of the pigment composition of all samples, showing Axes 1 and 2 (eigenvalues 0.70 and 0.24, respectively). (a) Sample scores for phytoplankton samples (Phyto) and copepod samples (Cop). (b) Pigment scores, ◇ = 13 pigments unique to the phytoplankton samples (some scores overlapping), △ = 14 pigments unique to the copepod samples, ● = 12 pigments found in both types of samples. Pigment abbreviations: Allo = alloxanthin; cf Allo = alloxanthin-like; Anthera = antheraxanthin; Asta = astaxanthin; Beta = β,β-carotene, Beta-Eps = β,ε-carotene; B1, B2, B3 = different forms of β-carotene; Cantha = canthaxanthin, chl a = chlorophyll a; chl b = chlorophyll b; chl c = chlorophyll c; Croc = crocoxanthin; Di = diesters of astaxanthin; Diadino = diadinoxanthin; Diato = diatoxanthin; Echi = echinenone; Fuco = fucoxanthin; Hexa = 19′-hexanoyloxy-fucoxanthin; Lut = lutein; Lyco = lycopene; Mono = monoesters of astaxanthin; Peri = peridinin; Pheo = pheophorbide a; Prasino = prasinoxanthin; P1 and P2 = 2 unknown pigments in phytoplankton samples; Viola = violaxanthin; Zea = zeaxanthin; Z1–Z6 = 6 unknown pigments in copepod samples

also confirmed by microscopic observations of high abundances of diatoms belonging to the genera *Chaetoceros*, *Cylindrotheca*, *Guinardia*, *Leptocylindrus* and *Skeletonema*. Furthermore, the carotenoids alloxanthin, 19'-hexanoyloxy-fucoxanthin, peridinin and echinenone confirmed the microscopically observed occurrences of small-celled cryptophytes, prymnesiophytes, dinoflagellates and cyanobacteria

Table 2. Mean initial pigment concentrations (nmol l^{-1}) in the phytoplankton samples (n = 3, SD = standard deviation) for each of the 4 treatments NSW, NP, NPSi1 and NPSi2. RCA = relative carotenoid abundance, – = pigment not detected

	NSW Mean	± SD	(RCA %)	NP Mean	± SD	(RCA %)	NPSi1 Mean	± SD	(RCA %)	NPSi2 Mean	± SD	(RCA %)
Alloxanthin	0.73	± 0.02	11.1	0.87	± 0.00	3.6	0.07	± 0.02	0.1	1.09	± 0.17	2.7
Antheraxanthin	–			0.27	± 0.01	1.1	–			–		
β,β-carotene	0.46	± 0.03	7.0	2.29	± 0.00	9.5	3.93	± 0.20	5.3	2.23	± 0.32	5.5
β,ε-carotene	0.11	± 0.01	1.7	0.24	± 0.02	1.0	–			0.14	± 0.03	0.3
Diadinoxanthin	0.36	± 0.02	5.5	1.56	± 0.06	6.5	12.39	± 0.18	16.6	4.30	± 0.62	10.7
Diatoxanthin	0.04	± 0.01	0.6	0.12	± 0.04	0.5	0.40	± 0.01	0.5	0.13	± 0.02	0.3
Echinenone	–			0.23	± 0.01	0.9	2.30	± 0.21	3.1	0.21	± 0.13	0.5
Fucoxanthin	1.88	± 0.02	28.5	5.09	± 0.08	21.0	48.86	± 2.07	65.6	28.55	± 3.90	70.8
19'-hexanoyloxy-fucoxanthin	0.05	± 0.02	0.8	–			0.86	± 0.04	1.2	0.53	± 0.06	1.3
Lutein	0.63	± 0.01	9.6	5.03	± 0.21	20.8	0.84	± 0.05	1.1	0.38	± 0.07	0.9
Peridinin	0.14	± 0.02	2.1	1.12	± 0.04	4.6	1.30	± 0.05	1.7	0.74	± 0.15	1.8
Prasinoxanthin	0.12	± 0.01	1.8	–			–			–		
Violaxanthin	0.34	± 0.02	5.2	1.99	± 0.05	8.2	0.84	± 0.03	1.1	0.46	± 0.06	1.2
Zeaxanthin	0.88	± 0.01	13.4	1.54	± 0.15	6.4	1.18	± 0.02	1.6	0.17	± 0.03	0.4
Other carotenoids	0.85	± 0.11	12.9	3.86	± 0.08	15.9	1.51	± 0.28	2.0	1.40	± 0.39	3.5
Chl *a*	7.37	± 0.05		30.73	± 0.65		59.46	± 1.82		36.41	± 5.33	
Chl *b*	1.56	± 0.07		5.65	± 0.10		0.80	± 0.04		1.04	± 0.11	
Chl *c*	1.70	± 0.18		7.10	± 0.14		62.31	± 3.75		44.30	± 5.10	
Pheophorbide *a*	0.04	± 0.02		–			0.13	± 0.00		–		

Table 3. Significant (p < 0.05) linear regressions with incubation time (h) as predictor variable and pigment concentrations of the phytoplankton (pmol l^{-1}) as response variables for each of the 4 treatments NSW, NP, NPSi1 and NPSi2 (n = 39 measurements), rc = regression coefficient, ns = not significant, – = pigment not detected

	NSW rc (R^2)		NP rc (R^2)		NPSi1 rc (R^2)		NPSi2 rc (R^2)	
Alloxanthin	−5.7	(0.71)	−8.1	(0.58)	ns		−14.8	(0.36)
Antheraxanthin	–		−2.9	(0.23)	–		–	
β,β-carotene	ns		−18.0	(0.39)	ns		ns	
β,ε-carotene	ns		ns		–		ns	
Diadinoxanthin	−5.3	(0.74)	−31.9	(0.87)	−100.1	(0.27)	ns	
Diatoxanthin	−0.6	(0.25)	−3.6	(0.55)	+3.7	(0.13)	−4.8	(0.49)
Echinenone	–		+1.8	(0.13)	ns		ns	
Fucoxanthin	−6.0	(0.13)	−75.5	(0.75)	ns		ns	
19'-Hexanoyloxy-fucoxanthin	ns		–		−19.6	(0.43)	−7.6	(0.18)
Lutein	−5.5	(0.61)	−40.5	(0.58)	−6.1	(0.11)	−3.8	(0.29)
Peridinin	−1.0	(0.20)	−26.7	(0.88)	ns		ns	
Prasinoxanthin	−1.8	(0.45)	–		–		–	
Violaxanthin	−3.2	(0.52)	−17.0	(0.59)	+7.2	(0.25)	−2.0	(0.15)
Zeaxanthin	−11.9	(0.75)	−13.4	(0.53)	ns		ns	
Chl *a*	−47.3	(0.46)	−339.5	(0.63)	ns		−219.6	(0.18)
Chl *b*	−14.7	(0.42)	−54.1	(0.53)	ns		−11.4	(0.43)
Chl *c*	−11.5	(0.27)	−125.0	(0.71)	ns		ns	
Pheophorbide *a*	+1.5	(0.26)	–		+3.7	(0.40)	–	

(*Merismopedia*, *Microcystis*, *Anabaena*), respectively. The phytoplankton pigment composition of the NSW and NP treatments, together with microscopic observations, revealed a mixture of chlorophytes (chl *b*, lutein, violaxanthin, antheraxanthin, zeaxanthin), particularly *Planktonema* (Ulotrichales) and *Scenedesmus* and other Chlorococcales, diatoms represented mainly by *Skeletonema* (fucoxanthin, diadinoxanthin), dinoflagellates (peridinin), cryptophytes (alloxanthin) and cyanobacteria (zeaxanthin, echinenone). Major differences between the 4 treatments were the much lower relative abundances and visibly thinner silica shells of the diatoms, and the much higher relative abundances of chlorophytes in the NSW and NP treatments compared with the 2 NPSi treatments.

Trends indicative of selective grazing during the experiments were shown by linear regression analysis of the pigments in the phytoplankton communities with time as predictor variable (Table 3). However, it should be kept in mind that these trends only represent a rough measure of grazing, because they summarise a complex combination of grazing and algal growth factors. In the NSW and NP treatments the copepods exploited a more diverse diet (negative regression coefficients for 12 and 13 pigments, respectively) than in the NPSi1 and NPSi2 treatments (negative regression coefficients for 3 and 7 pigments, respectively). The largest negative regression coefficients for carotenoids were found for fucoxanthin, diadinoxanthin, lutein and peridinin in the NP treatment and for diadinoxanthin and 19′-hexanoyloxy-fucoxanthin in the NPSi1 treatment (Table 3). These results suggest significant selective grazing on diatoms, chlorophytes and dinoflagellates in the NP treatment, and on prymnesiophytes in the NPSi1 treatment, while grazing rates were very low in the other 2 treatments as well as on other algal groups in the NP and NPSi1 treatments. Of the carotenoids reported to be precursors of astaxanthin, lutein had significant negative regression coefficients in all treatments, albeit 7 to 11 × lower in the 3 other treatments than in the NP treatment. This observation underlines the importance of chlorophytes as prey for copepods. Significant regression coefficients for zeaxanthin were found in the NP and NSW treatments, but for β,β-carotene only in the NP treatment. Five positive regression coefficients were also found (Table 3); these may be indicative of growth of a specific algal group, carotenoid dynamics such as xanthophyll cycling or copepod faecal pellets (pheophorbide *a*). However, all 5 positive regression coefficients were very small and therefore of no or negligible importance in the present study.

The zooplankton density (ind. l^{-1}; mean ± SD) in the 4 experiments was 49 ± 3 copepods and no other zooplankton in the NSW treatment, 41 ± 17 copepods and

3 ± 1 other zooplankton in the NP treatment, 41 ± 8 copepods and no other zooplankton in the NPSi1 treatment, 68 ± 6 copepods and 3 ± 1 other zooplankton in the NPSi2 treatment. Other zooplankton organisms were all more than 10 × smaller in body size than the copepods and were ignored in the calculations. The dominant calanoid copepod genus in all experiments was *Acartia*, with relative abundances of 89% (NSW), 99% (NP), 98% (NPSi1) and 95% (NPSi2) of all copepods. The main accompanying copepod genus was *Temora*, but single *Eurytemora*, *Paracalanus* and *Pseudocalanus* species were observed as well.

In July, the initial pigment content per copepod individual of the wild copepods (Fig. 3, NP and NPSi1, *t* = 0 h) was generally higher than in September (Fig. 3, NSW and NPSi2, *t* = 0 h). The major carotenoids in the copepod samples of the NPSi treatments consisted of ~8% of algal carotenoids that are involved in the biosynthesis of astaxanthin (β-carotene, zeaxanthin/lutein), ~68% of other algal carotenoids (fucoxanthin, diadinoxanthin, 19′-hexanoyloxy-fucoxanthin) and ~24% of zooplankton carotenoids (free astaxanthin, monoesters of astaxanthin, diesters of astaxanthin, alloxanthin-like). The major carotenoids in the copepods of the NSW and NP treatments consisted of ~17% of algal carotenoids that are involved in the biosynthesis of astaxanthin (β-carotene, zeaxanthin/lutein), ~26% of other algal carotenoids (fucoxanthin, diadinoxanthin, peridinin, violaxanthin) and ~57% of zooplankton carotenoids (free astaxanthin, mono- and diesters of astaxanthin, alloxanthin-like). β-carotene and zeaxanthin/lutein, as well as the typical zooplankton carotenoids free astaxanthin, monoesters of astaxanthin and alloxanthin-like, showed maxima in the early morning hours (before or just after sunrise) in the NP and NPSi1 treatments (Fig. 3). In the NSW and NPSi2 treatments maxima were less pronounced or absent. Zeaxanthin/lutein, astaxanthin and alloxanthin-like were highest in the copepods in the NP treatment, while β-carotene and monoesters of astaxanthin were in the same order of magnitude in the NP and NPSi1 treatments. The higher abundance of algal pigments in the 2 NPSi treatments depended on the higher abundance of larger diatom cells in colonies that became captured on the 200 µm mesh size plankton net together with the copepods while sampling.

In the NSW treatment, algal groups with diadinoxanthin (positive TR$_{net}$) and no or little fucoxanthin (negative TR$_{net}$) were preferred as prey by the copepods (Fig. 4a). A more varied diet was observed in the NP treatment, with positive TR$_{net}$ values for peridinin, violaxanthin, fucoxanthin and diadinoxanthin throughout most of the experiment (Fig. 4c). In both NPSi treatments the copepods showed the strongest
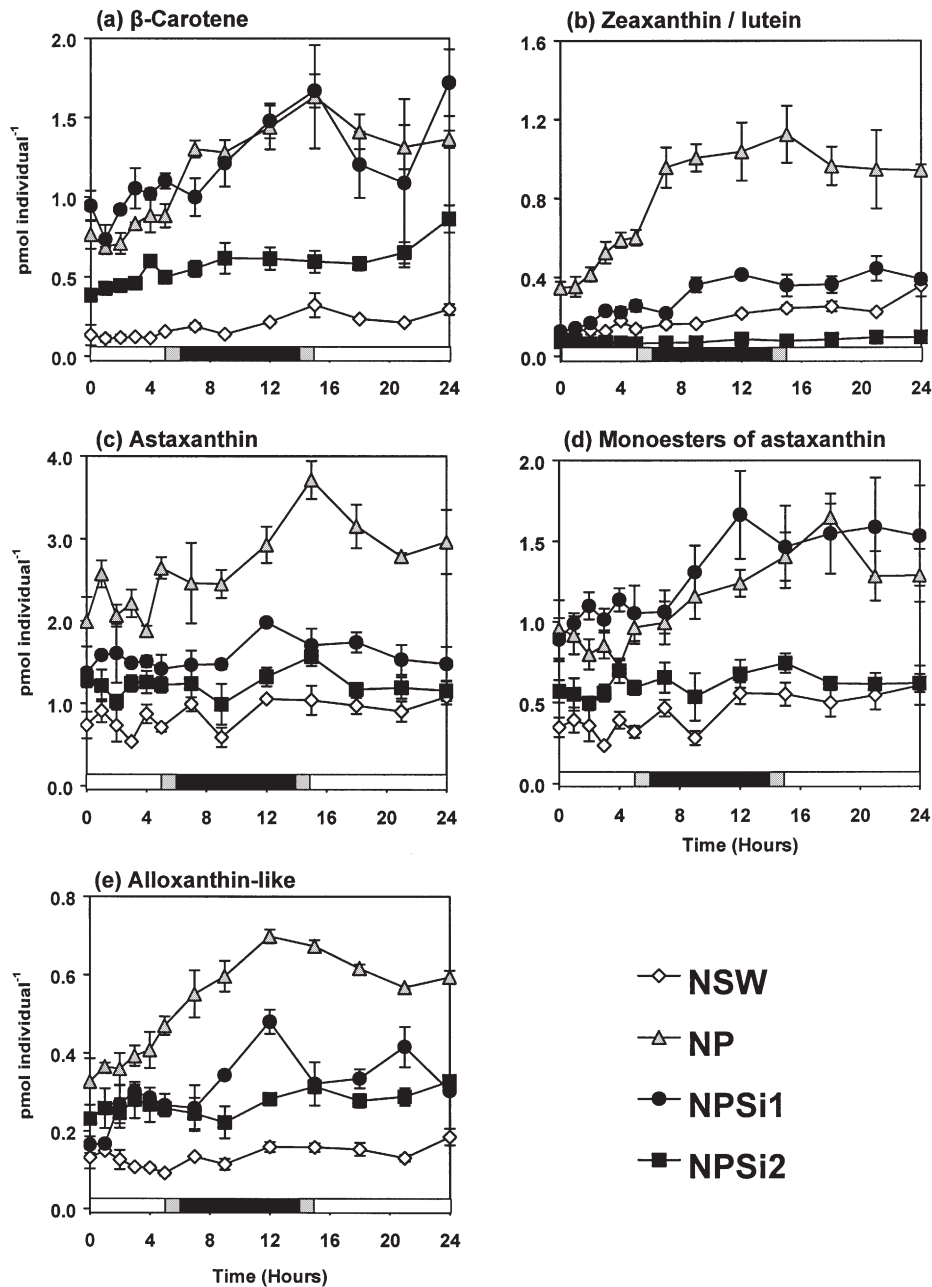
Fig. 3. Content of carotenoids in the calanoid copepods during the 4 treatments NSW, NP, NPSi1 and NPSi2, expressed as pmol per copepod individual. (a) Total β-carotene, (b) zeaxanthin/lutein, (c) astaxanthin, (d) monoesters of astaxanthin, (e) alloxanthin-like. Note the different scales on the y-axes. The x-axis shows the light period (white + grey bars) and the dark period (black bar) during the NP and NPSi treatments in July, and the light period (white bar) and dark period (black + grey bars) during the NSW and NPSi2 treatments in September. Error bars show mean ± 1 SE

preference for 19′-hexanoyloxy-fucoxanthin containing algae (prymnesiophytes) (Fig. 4e,g). Violaxanthin, fucoxanthin and diadinoxanthin had also positive TR_{net} values (around 0.2) in the NPSi1 treatment, as well as violaxanthin (up to ca. 0.5) in the NPSi2 treatment. In the NPSi2 treatment, selection was against algae containing peridinin (dinoflagellates).

Carotenoids reported to be involved in the biosynthesis of astaxanthin, total β-carotene (β,β-carotene, β,ε-carotene and their cis-forms) and zeaxanthin/lutein (the HPLC column used for the copepods samples could not separate these 2 carotenoids) were abundant in both phytoplankton and copepod samples. The TR_{net} was calculated for total β-carotene and for zeaxan-
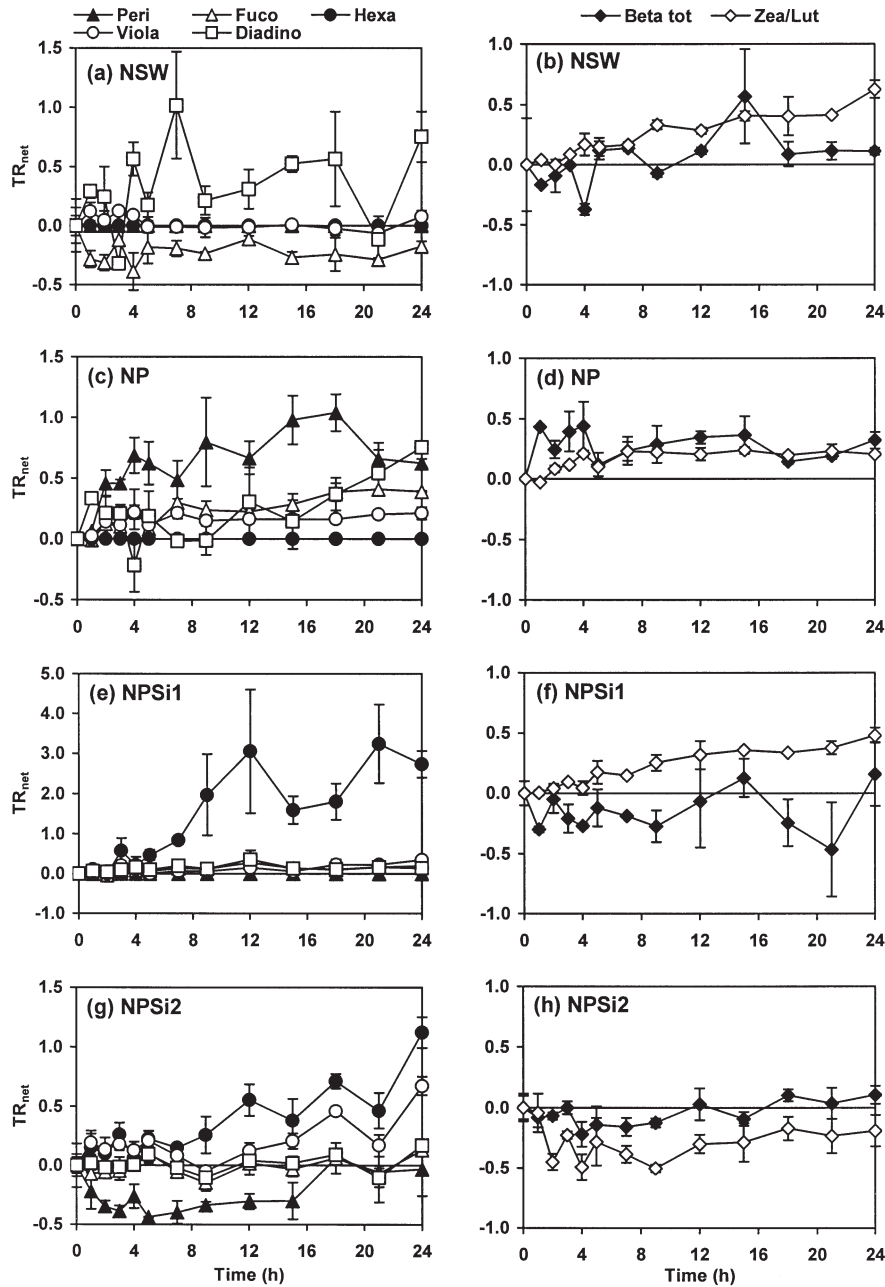
Fig. 4. Change in transfer ratios (TR_{net}) throughout the experiments for the 4 treatments NSW, NP, NPSi1 and NPSi2 for the carotenoids total β-carotene (Beta), diadinoxanthin (Diadino), fucoxanthin (Fuco), 19′-hexanoyloxy-fucoxanthin (Hexa), peridinin (Peri), violaxanthin (Viola) and zeaxanthin/lutein (Zea/Lut). Note the different scales on the *y*-axes. Error bars show mean ± 1 SE

thin/lutein. In the NP treatment TR_{net} values for total β-carotene were high throughout the experiment (Fig. 4d), suggesting accumulation of total β-carotene in the copepods. The TR_{net} values for total β-carotene were generally lower in the NSW treatment than in the NP treatment and lowest in the NPSi treatments (Fig. 4b,f,h), but maxima occurred at *t* = 15 h in the

NSW and NPSi1 treatments. The TR_{net} value for zeaxanthin/lutein increased steadily in the NSW, NP and NPSi1 treatments, but was negative throughout the NPSi2 treatment. The increases in zeaxanthin/lutein may have resulted from biosynthesis (from β-carotene), but also from selective grazing on chlorophytes.

DISCUSSION

In our experiments the calanoid copepods fed selectively on the phytoplankton in the different nutrient treatments. One obvious trend was that they did not graze on diatoms in the 2 NPSi treatments where diatoms dominated, while diatoms were among the major food items in the NP treatment. Some authors, e.g. Kleppel et al. (1991), have previously reported copepod grazing selectivity against diatoms, while others found that copepods feed selectively on diatoms (Meyer-Harms et al. 1999, Gasparini et al. 2000). It has also been found that copepods sometimes do not feed selectively at all (Kleppel 1998). Based on studies of growth, fecundity and egg mortality of copepods, it may be true that only fast-growing and nutrient-replete diatom cells are high-quality food for copepods, while senescent cells and cells with a high C:N ratio have a poor food quality (Kiørboe 1989, Jónasdóttir 1994). In a previous, similar mesocosm experiment carried out on the Norwegian Atlantic coast, we showed that the phytoplankton in the NPSi treatment (completely dominated by diatoms) suffered from N-limitation (Van Nieuwerburgh et al. unpubl.), which may have affected food preference dynamics. Both in the earlier and the present studies, the reason for the copepods avoiding consumption of diatoms in the NPSi treatments may also have been that the diatoms in these treatments were exceptionally heavily silicified, and therefore difficult to eat.

In the present study, we tested how the production of the important antioxidant astaxanthin in calanoid copepods is affected when they graze on different phytoplankton communities. It is generally recognised that the main precursor of astaxanthin in zooplankton is β,β-carotene (Katayama et al. 1973, Bandaranayake & Gentien 1982). It has also been demonstrated that zeaxanthin is metabolised into astaxanthin via β-doradexanthin, while alloxanthin and diatoxanthin can be transformed into didehydroastaxanthin and tetradehydroastaxanthin, respectively (Ohkubo et al. 1999). Lutein, a carotenoid frequently reported as common in crustaceans, is converted into α-doradexanthin, but there is no conclusive evidence concerning a further conversion into its isomer, β-doradexanthin, leading to the formation of astaxanthin (Ohkubo et al. 1999). However, astaxanthin biosynthesis from lutein has been suggested to occur in the ovaries of the crayfish *Cherax quadricarinatus* (Sagi et al. 1995), as well as in developing eggs of the crayfish *Astacus leptodactylus* (Berticat et al. 2000). This would imply that lutein might also act as a precursor of astaxanthin in crustaceans.

Our results suggest that the quality of the phytoplankton community as food for calanoid copepods

depends both on the quantity and the quality of the food items (biomass and community composition). Despite a similar phytoplankton pigment composition in the NSW and NP treatments (as we showed by PCA; Fig. 2), the NP treatment yielded a much higher astaxanthin production in the copepods in our experiments (Fig. 3). This could be attributed to the ~5× higher phytoplankton biomass in the NP treatment; the relative abundance of β-carotene is similar (NSW: 7.0%, NP: 9.5%) in the 2 treatments, but the total β-carotene available to the copepods was 5× higher in the NP treatment (Table 2). Moreover, other presumed precursors of astaxanthin, lutein and zeaxanthin available to the copepods were 8 and 2× higher in the NP treatment, respectively. Thus, a higher availability of chlorophytes in the NP treatment, next to diatoms and dinoflagellates, may have been advantageous for the production of astaxanthin in addition to the higher biomass. Both NPSi treatments had higher biomass than the NP treatment (Table 2), but lower astaxanthin production (Fig. 3), which suggests that the quality of the available prey is more important for astaxanthin production than biomass per se. Astaxanthin production was higher in the NPSi1 treatment than in the NPSi2 treatment (Fig. 3), especially the production of astaxanthin monoesters. The pigment composition of the phytoplankton in the 2 NPSi treatments was very similar (as we showed by PCA; Fig. 2), but there were some other differences which may have affected both grazing and astaxanthin production: (1) the NPSi1 treatment had a factor 1.5 higher biomass based on chls *a* + *c* (Table 2), and thus a relatively higher access to β-carotene, lutein and zeaxanthin, (2) higher consumption of prymnesiophytes in NPSi1 treatment (Table 3), (3) seasonal differences in life cycle and/or physiology of the copepod community between July (NPSi1) and September (NPSi2), and (4) the difference in copepod density between NPSi1 and NPSi2 (41 and 68 ind. l^{-1}, respectively). The transfer ratios of β-carotene were similar for the 2 treatments, but that of zeaxanthin/lutein was positive for the NPSi1 treatment and negative for the NPSi2 treatment. This suggests that the difference in astaxanthin production is mainly related to the transfer of astaxanthin precursors.

Since planktonic crustaceans cannot synthesise carotenoid pigments de novo Matsuno (1989), and because zooplankton body pigments are not normally present in phytoplankton algae, our observed variations in astaxanthin and alloxanthin-like carotenoid content in the copepods provide indications of diurnal variation in body pigment metabolism. In our experiments, the concentrations of copepod body carotenoids peaked around sunrise (Fig. 3). The most

pronounced peak in astaxanthin content per copepod individual occurred in the NP treatment, with levels about twice the initial value. The occurrence of these peaks is probably driven by increased feeding activity during the night because also β-carotene and zeaxanthin/lutein showed the same trends. A diurnal feeding pattern in zooplankton was described by Kleppel et al. (1985) from Los Angeles Harbor, California, with peaks for both feeding activity and total carotenoid content during the night. The relationship between total carotenoid content and feeding activity could be described by a linear regression model. Also Stearns (1986) revealed a nocturnal feeding pattern in *Acartia tonsa* Dana and a negative correlation between feeding and light intensity during daylight hours in a North Carolina estuary. Based on these results and laboratory experiments, Stearns (1986) hypothesised that feeding might be a behaviour controlled by light separate from copepod migration. A photoprotective role of astaxanthin in copepods was suggested by Hairston (1979a,b), and the rationale for the periodicity we discovered in our study may be that astaxanthin is accumulated by the zooplankton during the night and utilised for photoprotection and other antioxidant activities during the day.

Our experiments were short-term and were carried out in the laboratory. Therefore the results cannot directly be translated to nature, despite the use of natural communities of phytoplankton and copepods. However, some of the results we obtained may play a part in complex, naturally occurring processes. In this study, we showed that phytoplankton community composition and biomass have profound effects on the production of the vital compound astaxanthin in calanoid copepods. Zooplankton occupies a key position in the pelagic food web as it transfers the organic energy produced by unicellular algae through photosynthesis to higher trophic levels such as pelagic fish stocks. The important role of calanoid copepods in food webs worldwide (Mauchline 1998) implies that the whole food web may be affected by the quality and quantity of the phytoplankton as prey for higher trophic levels. With a more varied diet and high biomass (NP treatment) astaxanthin production in the copepods was highest. Astaxanthin production was much lower with high phytoplankton biomass dominated by large heavily silicified diatoms (NPSi treatment). These results suggest a positive effect of eutrophication, but not when excess Si is available for phytoplankton growth. Large-scale and long-term eutrophication of the Baltic Sea may lead to lower phytoplankton diversity, with blooms of 1 or a few species, and thus a less varied diet for zooplankton, which in the light of our experiments may lead to a lower production of astaxanthin in the food web.

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