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Growth phase of the diatom *Skeletonema marinoi* influences the metabolic profile of the cells and the selective feeding of the copepod *Calanus* spp.

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Copepods dominate the biomass of marine zooplankton and through their prey selection they act as top-down regulators of planktonic communities. We investigated feeding preference of copepods in the presence of the diatom *Skeletonema marinoi* at different time points throughout the development of a bloom and a culture. Quantitative PCR gut content assessment revealed that the food uptake of the copepod *Calanus* spp. on mixed diets and on artificially induced mesocosm blooms was selective. Uptake of *S. marinoi* was highest during the post-bloom phase in the mesocosms even if the abundance of this alga was already low. In laboratory assays, copepods showed a greater preference for *S. marinoi* in the late stationary phase than for cultures of the same strain under exponentially growing culture conditions. The copepods thus discriminate between different growth phases of a single algal species in both laboratory and field settings. In parallel, we monitored cellular metabolites of the diatom using a metabolomic approach. Complex changes in the metabolic profile occur during development of a culture. Since no obvious effect of nutrient quality and cell size was involved, we suggest that changes in (info)chemicals within or surrounding *S. marinoi* regulate selective feeding by zooplankton.

INTRODUCTION

Calanoid copepods are planktonic crustaceans that play important roles in marine food webs. As the primary prey for many juvenile and adult fish, they are key mediators of energy transfer to higher tropic levels. Copepods are also known to shape the microbial food web through their selective grazing activity. Until the 1970s, with only a few notable exceptions (Harvey, 1937), predation by copepods was considered a non-selective process involving a random foraging behavior, mechanistically responding to factors such as prey size and concentration (e.g. Frost, 1972). Since then, evidence has accumulated on the selective foraging by copepods showing that both mechano- and chemoreception is involved in the evaluation of suitable food particles before ingestion (Kleppel, 1993). Early studies by Poulet and Marsot (Poulet and Marsot, 1978) demonstrated the importance of chemoreception in the feeding of calanoid copepods using artificial encapsulated food with different chemical cues. Subsequently, a series of reports documented that

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chemoreception is involved in the detection of toxins, as well as in the location and evaluation of food particles. Huntley *et al.* (Huntley *et al.*, 1986), for example, reported that chemical cues from dinoflagellates can induce feeding avoidance in *Calanus pacificus*. Copepods can also decrease their feeding activity if exposed to toxins (Breier and Buskey, 2007; Waggett *et al.*, 2008). If alternative food is present, toxic prey can be avoided in favor of non-toxic food particles (Makino *et al.*, 2008). Copepods even exhibit differential feeding activity if *Alexandrium minutum* diets of the same isolate with different toxin levels are offered (Selander *et al.*, 2006).

In addition to the ability of copepods to change feeding behavior in response to various toxins, copepods also react to food particle quality with differential food uptake (Huntley *et al.*, 1983; Mayzaud *et al.*, 1996; Olson *et al.*, 2006). The chemosensory capabilities of copepods are also demonstrated by their use of chemical signals for remote prey location or mate finding (Moore *et al.*, 1999, Steinke *et al.*, 2006). Chemical signals affecting copepods thus have a major impact on plankton ecology and plankton dynamics.

Several factors, such as food quality and toxicity, are highly plastic in algae. Depending on nutritional resources, various stress factors, age and clone, cells of one single species might significantly differ in biochemical composition (Malej and Harris, 1993; Jonasdottir, 1994; Wichard *et al.*, 2005; Selander *et al.*, 2006). Feeding strategies that rely solely on the selection of prey at the species level might therefore not be sufficient. Algae that are good food source under certain growth conditions can decrease in nutritional value, which ultimately affects the fitness of copepods (Jonasdottir, 1994). Recognition of diatoms in different growth phases might rely on mucus produced predominantly in the stationary phase (Malej and Harris, 1993). However, variation in more than mucus secretion is observed. The amount and nature of many other compounds released by diatoms is highly variable and depends on the age and state of cultures (Barofsky *et al.*, 2009). As a result, a highly complex chemical sphere builds up around phytoplankton cells. Since this phycosphere changes over time, it has the potential to transmit information on the state of a cell to the copepods (Moore *et al.*, 1999).

In this study, we investigated whether variability in chemical profiles of diatoms during different growth phases relates to variable copepod ingestion. We evaluated the impact of the common diatom *Skeletonema marinoi* in different growth phases on the food selection of the common copepod *Calanus* spp. The test organisms co-occur in nature and shape the plankton community, especially during spring blooms. We first conducted a mesocosm study in which we induced a *S. marinoi* bloom and followed food ingestion by *Calanus* spp. The amount of *S. marinoi* in the gut of copepods did not peak during the phase of maximum prey cell counts in the mesocosm. In a controlled laboratory feeding experiment, the selective food uptake depended on the developmental phase of the culture. In addition, we performed metabolic profiling analyses, revealing that chemical profiles of the cells and their exudates differed throughout the culture development.

METHOD

Mesocosm experiment

The experiments were conducted between the 14th and 28th of April 2008 at the Espegrend marine biological field station, Raunefjord, Western Norway (latitude 60.161°N; longitude: 5.138 °E). For a general description of the location, mesocosm design, filling procedures and water column mixing see Nejstgaard *et al.* (Nejstgaard *et al.*, 2006) and www.bio.uib.no/lsf/inst2.html. In short, on 14th April transparent polyethylene enclosures (each 11 m^3, 2 m diameter, open to air) were filled with unfiltered sea water from 4 m depth just outside the mesocosms, and the following day the mesocosms 1 and 2 were fertilized. Mesocosm 1 had a starting concentration of 0.36 μmol L^{-1} phosphate, 4.23 μmol L^{-1} nitrate and 3.42 μmol L^{-1} silicate after fertilization, and was inoculated with cultured *S. marinoi* to reach a final cell count of ca. 100 cells mL^{-1}. Mesocosm 2 had a starting concentration of 0.4 μmol L^{-1} phosphate, 4.24 μmol L^{-1} nitrate and 3.61 μmol L^{-1} silicate after fertilization, and was inoculated with a final cell count of ca. 400 *S. marinoi* cells mL^{-1}. Water temperature was measured daily throughout the water column in the mesocosms with a SAIV SD204 CTD.

The abundance of *S. marinoi* was determined daily using a CytoBuoy scanning flow cytometer (http://www.cytobuoy.com/). The CytoBuoy flow cytometer is particularly useful in quantifying abundance and chain length of diatoms like *Skeletonema* (Takabayashi *et al.*, 2006). Based on the lower per cell fluorescence and the presence of repetitive cell units in chains, *S. marioni* was separated from the ambient phytoplankton community. Assuming a constant cell length, the number of cells in each chain was estimated and in turn summarized for the entire sample. Additional general description of the analysis and results from the mesocosm experiment will be presented elsewhere (Nejstgaard *et al.*, unpublished results). Protozoans were enumerated using a B/W FlowCAM II (Sieracki *et al*, 1998) which is an image in flow automated microscope capable of quantifying plankton particles. We acquired particle images with a 4x objective in auto-imaging mode. The total processed volume per sample

was 4 mL. After data acquisition, particles were identified and sorted into species or groups. We identified the most important alternative prey and divided them into four groups namely ciliates, *Protoperidinium bipes*, *Gymnodinium dominans* and *Gyrodinium spirale*. Biovolume was calculated from the area-based diameter algorithm of the FlowCAM and expressed as ppm. The biovolume of *S. marinoi* was estimated with a Multisizer III Coulter Counter using a similar *S. marinoi* laboratory grown clone (J4) that only produces single cells.

Phytoplankton cultures

Algal inoculation culture for the mesocosms was the diatom *S. marinoi* University of Bergen, Department of Biology strain G4 (isolated on 13 June 2006 at the permanent station in the Raunefjord, one nautical mile off the field station). Mesocosm inoculation cultures were grown in 10 L clear polyethylene bags containing f/2 medium supplemented with silicate (Guillard, 1975) in a 14:10 light:dark cycle and bubbled with sterile filtered air. For metabolic profiling, the strain *S. marinoi* G4 was grown in triplicate in standing 25 L polycarbonate bottles at 15°C in 25 L artificial medium (Maier and Calenberg, 1994). For inoculation procedures see Vidoudez and Pohnert (Vidoudez and Pohnert, 2008). The bottles were bubbled with sterile filtered air and were equipped with a liquid and air outlet. A dripping chamber allowed sterile sampling (Vidoudez and Pohnert, 2008). Illumination was provided from the side with a 14:10 light:dark regime, with a light intensity of 45 μmol m^{-2} s^{-1} measured at the outside surface of the culture bottle. Cell density was estimated by counting at least 400 cells in a Fuchs–Rosenthal hematocytometer using an upright microscope. As control, a 25 L polycarbonate bottle was filled with 25 L medium and treated with exactly the same protocol as the cultures.

For the laboratory feeding experiments, *S. marinoi* G4 and *Rhodomonas marina* were grown in batches using f/2 medium supplemented with silicate (Guillard, 1975). *Skeletonema marinoi* was grown at 9°C and *R. marina* at 14°C. Both algae were maintained in a light:dark regime of 14:10 h. *Isochrysis* sp. was cultured as described in Bouquet *et al.* (Bouquet *et al.*, 2009). *Skeletonema marinoi* was kept in exponential phase by 1:5 dilution of the medium every other day. *Rhodomonas marina* and *Isochrysis* sp. cell concentrations were determined by Coulter Counter while *S. marinoi* cells were counted in a Fuchs–Rosenthal hematocytometer using an upright microscope.

Copepod sampling and mesocosm grazing experiments

Four feeding experiments were performed during the last 7 days of the mesocosm experiment. For each experiment, copepods were collected approximately one nautical mile west of the mesocosm location. A 1 m diameter net with 333 μm mesh size and a 4 L non-filtering cod-end was towed obliquely from 50 to 0 m depth. Upon arrival at the laboratory, 20 copepodite stage-V (CV) females of *Calanus* spp. were sorted into 500 mL cylindrical acrylic Perspex chambers and further closed at both ends with two 500 μm nets. The chambers were suspended inside each mesocosm at 1 m depth for 3 days which allowed the copepods to feed at *in situ* conditions before they were harvested. Copepods were sampled according to a protocol that ensures minimal prey DNA digestion (Simonelli *et al.*, 2009). After the incubation, copepods were removed and quickly rinsed by dipping the Perspex chambers in three consecutive baths of 450-mL filtered (1 μm) seawater to wash off external prey particles. Copepods were then sedated by dipping the Perspex chamber in 400 mL 0.37 mg/mL tricaine methane sulphonate (MS222) (Sigma). The chambers were opened and the animals collected into Petri dishes containing 0.37 mg/mL MS222 and randomly sorted under dim light into 1.5 mL Eppendorf™ microtubes for DNA gut content analysis. Three samples of five *Calanus* spp. CV were collected from each mesocosm. To minimize the risk of inclusion of prey algae in the water on the outside of the copepods, they were sorted by grabbing the base of the antennules with a forceps and dip-washed in droplets of prey-free filtered seawater before transfer to the tubes. Care was taken to minimize the amount of water in the tubes. For analysis of prey DNA content 100 mL of mesocosm water was collected after each feeding experiment. Water was filtered onto 0.2 μm Supor® filters (Pall Life Sciences) and the filters were placed in 1.5 mL Eppendorf™ microtubes for DNA analysis.

Laboratory grazing experiments

Copepods were collected as described earlier. Plankton tow samples were diluted in 40 L surface water and transferred within 30 min after collection to a walk-in cold room, maintained at constant temperature (10°C) with dim light on a 14:10 h light:dark regime. Copepodites CV females of *Calanus* spp. were sorted into 5 L beakers containing 1 μm filtered and UV sterilized sea water using a wide mouth pipette. Animals were acclimated for 14 days with a mixed diet of *R. marina*, *S. marinoi* G4 and *Isochrysis* sp. Total food concentration was maintained higher than 1600 μg C L^{-1} which is documented to yield saturated feeding rates by *Calanus finmarchicus* (Bamstedt *et al.*, 1999). Before the start of the experiment, copepods were randomly split into 10 groups of 11 individuals, transferred into

450 mL Perspex chambers with 500 μm false bottoms and incubated for 6 h with a suspension of 20 cells/μL of *Isochrysis* sp. to allow complete gut evacuation of *S. marinoi* and *R. marina*. Two initial samples of five copepods fed a diet of *Isochrysis* for 6 h were collected as a control.

Two different food suspensions were prepared using mixtures of 25 cells/μL of *R. marina* and 17 cells/μL of *S. marinoi*. In one of the two mixtures, *S. marinoi* was in exponential and in the other in stationary phase. Each of the food suspensions was split in five replicates and five groups of copepods were transferred into the food suspension containing *S. marinoi* G4 in exponential growth phase and the other five groups into the one containing *S. marinoi* G4 in stationary phase. After 20 min of incubation, copepods were sampled for DNA gut content analysis as described for the mesocosm grazing experiments. Two samples of five *Calanus* spp. CV each were collected from each replicate.

To determine prey algal DNA content, 1 mL of water was collected in 1.5 mL Eppendorf™ microtubes from each food suspension. Algal cells were harvested by centrifugation (7000 *g*) for 15 min, supernatant was removed and algal pellets were processed for DNA analysis.

The temperature was at 10°C throughout the experiments, algal cultures were adjusted to this temperature for 24 h prior to the experiment.

Algal-specific qPCR primers

A *Skeletonema* sp. 18S rRNA gene-specific qPCR assay was designed and optimized for this study. Primers were designed using the primer design tool GeneTool-lite v. 1.0 (BioTools, Inc.), and primers were evaluated *in silico* and further empirically optimized for qPCR using the MJResearch Opticon2® real-time thermal cycler. The specificity of the primers was empirically tested in standard PCR assays using genomic DNA purified from several other algal species including the cryptophyte *Rhodomonas marina*, the bacillariophytes *Chaetoceros curvisetus*, *Phaeodactilum tricornutum*, *Prorocentrum micans*, *Pseudo-nitzschia longissima*, *Amphidinium carterae*, *Heterocapsa triquetera*, the chlorophytes *Brachiomonas submarina*, *Nannochloris* sp., the haptophytes *Chrysochromulina ericina*, *Emiliania huxleyi*, *Isochrysis* sp., *Phaeocystis pouchetii*, *Prymnesium parvum* and the prasinophyte *Tetraselmis suecica*. The potential for non-specific amplification by these primer sets of metazoan and prokaryotic genes was also evaluated. Genomic DNA from the copepods *Calanus* spp., the appendicularian *Oikopleura dioica* and from the bacteria *Escherichia coli*, were also tested with these primer sets. In addition, a previously designed 18S rRNA gene targeted PCR primer pair specific for the cryptophyte algal genus *Rhodomonas* was used (Troedsson *et al.*, 2009). Table I reports the sequences for each of these primer pairs, annealing conditions and expected amplicon size.

Gut content analysis

Gut content analysis was performed using nucleic acids as a biomarker for quantitative estimates (Troedsson *et al.*, 2007; Nejstgaard *et al.*, 2008). Prey DNA extraction was performed using the DNeasy® Blood & Tissue kit (Qiagen, Inc.) as described in Simonelli *et al.* (Simonelli *et al.*, in press). Copepods were sorted directly into sterile 1.5 mL Eppendorf™ tubes containing 180 μL of homogenization buffer (ATL buffer, Qiagen, Inc.) and 20 μL Proteinase K. The tubes were further incubated at 55°C overnight. Prey DNA extraction was then completed following manufacturer's instructions with the additional recommended RNase A treatment. Algal DNA from the water samples collected on filters or by centrifugation was extracted following the same protocol.

Real-time PCR reactions were performed using an Opticon2 MJ Research real-time thermal cycler. The primer sets used are described in Table I. Twenty-five microlitre reaction volumes contained 12.5 μL of 2 X QuantiTech SYBR Green Master Mix (Qiagen) and 0.3 μM of each primer. Amplification was performed as follows: initial template denaturation (95°C for 15 min); 40 amplification cycles (94°C for 30 s; specific annealing temperature for 30 s; 72°C for 30 s) and a final extension step (72° for 10 min). Finally, a melting profile was performed from 65°C to 95°C in increments of 0.5°C/2s. PCR grade water was used as a template for the negative control. To convert the C(t) values obtained from the qPCR analysis to number of algal cells, dilution series of extracted genomic DNA from a known

Table I: Primers used in this study

Primer[a]	Primer sequences (5′ to 3′)	Product length (bp)	Annealing temperature (°C)	Source
Skel 175F	ACC GCC GTG TTT ATT AGT A	70	55.0	This study
Skel 244R	TCG AAA GGT TAT TAT GAC T			
Rhod 97F	CGT TTA TTT GAT GGT CCC TTA	74	53.3	Troedsson *et al.*, 2009
Rhod 150R	GTC GGA CCT TTG TGC ATG TAT			

[a]Primer name includes direction and target location.

number of algae collected during the mesocosm and laboratory experiments were run as reference standards together with the copepod samples. This is important since the 18S rDNA copy number may differ with respect to the physiological condition of the algae in the different experiments (Nejstgaard *et al.*, 2008). Due to prey DNA digestion in the gut of the copepod (Nejstgaard *et al.*, 2008; Troedsson *et al.*, 2009), all cell number estimates are semi-quantitative and the absolute numbers are therefore not directly comparable between different algal species, so interpretations were made based on the relative values. All samples, standards and negative controls were run in at least two independent qPCR reactions and the mean of these estimates was used for statistical comparisons.

Diatom culture sampling for metabolite profiling

Samples for metabolic profiling were taken 8 h after the onset of illumination from the 25 L cultures. The first sampling of the 500 mL cultures was performed 3 days after inoculation. Subsequently, a volume between 500 and 50 mL, depending on the cell density, was collected daily from each culture. The sampling air outlet was closed and the liquid outlet was opened thus pushing the sample through a silicon tube into a 1 L glass bottle. A subsample of 1 mL was taken to determine cell density. Cells were separated from medium by GF/C-filtration. The cells were rinsed off the filter using 1 mL methanol (UPLC/MS grade, Biosolve, The Netherlands). The cells were extracted by vortexing in this solvent for 1 min. Afterwards, the sample was centrifuged for 3 min (13.200 rpm) and the supernatant was directly analysed with ultra performance liquid chromatography/mass spectrometry (UPLC/MS).

UPLC/ESI-MS analysis

A Waters Acquity Ultra Performance LC equipped with a 50 mm Acquity UPLC BEH C_{18} column (2.1 mm, 1.7 μm) at a column temperature of 30°C was used for separation. Analytical details are described in Barofsky *et al.* (Barofsky *et al.*, 2009).

Chemometric analysis

UPLC-MS raw data were analysed using the MarkerLynx V4.1 software (Waters, UK) as described in Barofsky *et al.* (Barofsky *et al.*, 2009). The resulting two-dimensional data were analysed using the principal component analysis (PCA) software implemented. Data were z-scored prior to PCA and variance scaled. The Eigen-values were calculated with the MarkerLynx extension software Simca-$P+12.0$. Highly loaded m/z-retention time pairs were extracted with the corresponding loadings plot. For relative quantification, the peak areas were integrated, normalized by the extraction volume and cell number. The relative quantification was performed with the QuanLynx V4.1 software (Waters, UK).

RESULTS

In the mesocosm experiment, both enclosures inoculated with *Skeletonema marinoi* showed a similar growth curve of the diatom (Fig. 1). However, despite similar shapes of the growth curves, the larger inoculation in mesocosm 2 (starting with 400 cells mL^{-1}, compared to 100 cells mL^{-1}) resulted in nearly 20x higher cell counts of this alga compared with mesocosm 1. In both mesocosms, cell counts of *S. marinoi* increased rapidly

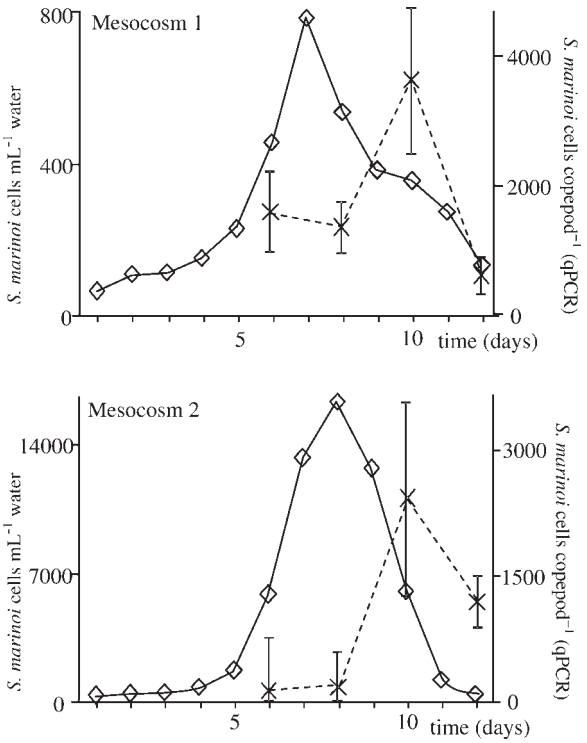

Fig. 1. Estimated *S. marinoi* counts in the mesocosm water (diamonds) and in the copepod gut (crosses) determined by quantitative PCR. Note that due to the semi-quantitative nature of the method (see Method), the cells detected by qPCR in the copepod gut do not necessarily correspond to the absolute cell number in the copepod gut. Mesocosm 1 was inoculated to reach an initial concentration of 100 *S. marinoi* cells mL^{-1} water and mesocosm 2 was inoculated to reach an initial concentration of 400 *S. marinoi* cells mL^{-1} water at day 1. Error bars indicate the standard deviation ($n=3$ for day 8, 10 and 12 and $n=2$ for day 6).

after day 4 reaching maximal cell concentrations of 780 cells mL^{-1} at day 7 in mesocosm 1 and 16 700 cells mL^{-1} at day 8 in mesocosm 2. The FlowCAM analysis revealed that alternative food was present for the copepods in the mesocosms throughout the experiment. The alternative prey, protozoans, could be separated into four groups namely ciliates, *Protoperidinium bipes*, *Gymnodinium dominans* and *Gyrodinium spirale*. However, because ciliates made 70% of the protozoan biomass, we finally pooled the dinoflagellates into one group (Fig. 2). The protozoan bio-volume increased in parallel in both mesocosms throughout the experiment from ca. 0.1 ppm on day 6 to ca. 0.4 ppm on day 12. Concerning the relative contribution of alternative protozoan prey, there was an increase in the protozoan over *S. marinoi* ratio in both mesocosms: In mesocosm 1, the ratio increased from ca. 1.7 at day 6 to 27 at day 12 and in mesocosm 2 from 0.15 on day 6 to 9 on day 12 (Fig. 2). Light microscopy confirmed the findings from the Flowcam. The water temperature in both mesocosms increased 1.1°C during the period the feeding experiments were conducted (from an average of 7.5 ± 0.0°C on day 6, to 8.6 ± 0.0°C on day 12).

The relative amount of *S. marinoi* cells ingested per copepod was estimated by qPCR gut content analysis in both mesocosms (Fig. 1). The development in *Calanus* spp. feeding was not directly correlated with the diatom biomass development, but the highest gut content estimates were recorded on day 10, which is 2–3 days after the maximal cell concentration in the water (Fig. 1). The ratio between the number of *S. marinoi* cells in the copepod gut and in the mesocosm water was significantly higher on day 10 than on day 8 both for mesocosm one (two-tailed *t*-test: $P = 0.021$) and mesocosm two (two-tailed *t*-test: $P = 0.038$). This indicates increased feeding on *S. marinoi* cells towards the breakdown of the bloom.

To minimize the multiple factors potentially influencing food uptake during mesocosm experiments, we developed a controlled laboratory food choice assay. This allowed us to specifically test the ability of *Calanus* spp. to distinguish between *S. marinoi* in different growth phases. *Calanus* spp. was fed different mixed diets composed either of *S. marinoi* in exponential growth phase and *Rhodomonas marina* or *S. marinoi* in stationary phase and *R. marina*. Initial cell concentrations of *S. marinoi* and *R. marina* were identical in both treatments. In these experiments, the copepods grazed significantly more on *S. marinoi* cells in the stationary phase, when compared with the exponentially growing culture (two-tailed *t*-test: $P = 0.002$, Fig. 3). The estimated relative cell number of the alternative prey *R. marina* detected in the gut was not significantly different between the copepods in the two experiments (two-tailed *t*-test: $P = 0.55$, Fig. 3). This suggests that the change in *Skeletonema* ingestion was not due to an overall change in feeding activity of the copepod.

We examined whether the variability of *Calanus* spp. food uptake is reflected in a variable intracellular

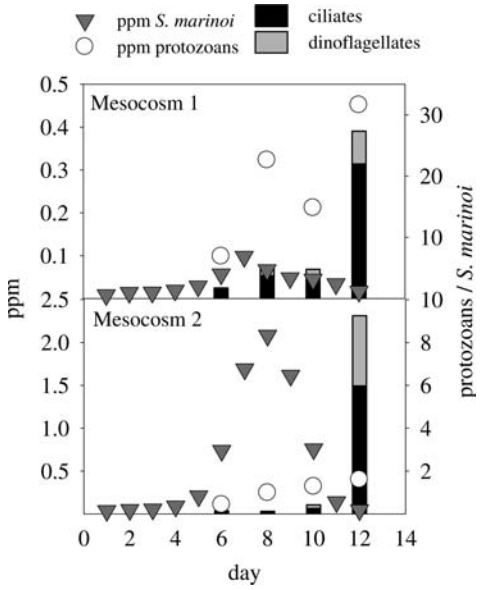

Fig. 2. Development of *Skeletonema marinoi* and alternative protozoan prey biovolume during the 12-day mesocosm experiment. Upper panel is mesocosm 1 and lower panel is mesocosm 2. Triangular labels refer to biovolume of *S. marinoi*, circular labels to biovolume of protozoans. The bar plots display the development in the ratio between protozoans and *S. marinoi* during the experiments. The left axis is biovolume and right axis is the biovolume ratio between protozoan and *S. marinoi*.

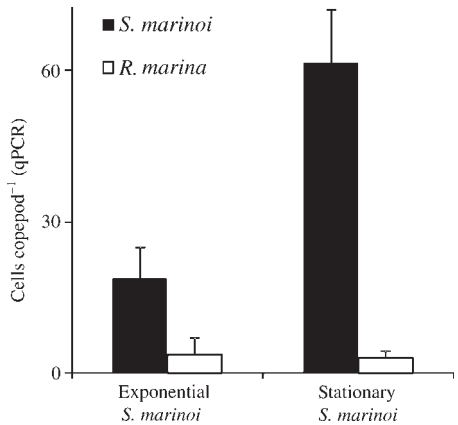

Fig. 3. Estimated *S. marinoi* and *R. marina* cell concentrations in *Calanus* spp. gut by quantitative PCR. Error bars indicate the standard deviation ($n = 3$). Note that due to the semi-quantitative nature of the method (see Method), the cells detected by qPCR in the copepod gut do not necessarily correspond to the absolute cell number in the copepod gut.

chemical profile of the algae. Different chemical profiles during different growth phases could reflect different nutritional or possible defensive properties. UPLC/MS profiling of whole cell extracts from 25 L batch cultures was performed daily during the exponential, stationary and declining phase of *S. marinoi*. UPLC/MS data were complex with several hundred mass/retention time pairs, each representing a specific metabolite. These data were interpreted using PCA. The score plot (Fig. 4) shows that the extracts of the exponential (triangle), the stationary (cross) and the declining (square) phase grouped separately. The first principal component has an Eigen-value of 19.9 and separated the exponential and the declining phase. The second principal component has an Eigen value of 12.9. Each replicate of the three independent *S. marinoi* culture extracts matched this pattern. By analysing the corresponding loadings plot, it is possible to identify metabolites responsible for the most pronounced changes of the chemical profiles. Among the hundreds of cellular metabolites, the highly loaded signals (corresponding to metabolites that exhibit the most significant changes) were from nine metabolites specifically produced by *S. marinoi* in declining phase and two produced in exponential phase. These 11 signals represent the most dynamically changing metabolites, which, according to a first data evaluation, are not standard lipids or saccharides. The differences in the metabolic profile among different days during culturing can be visualized by plotting the relative concentration of metabolites over the time. Such exemplary kinetics of two unidentified substances is given in Fig. 5.

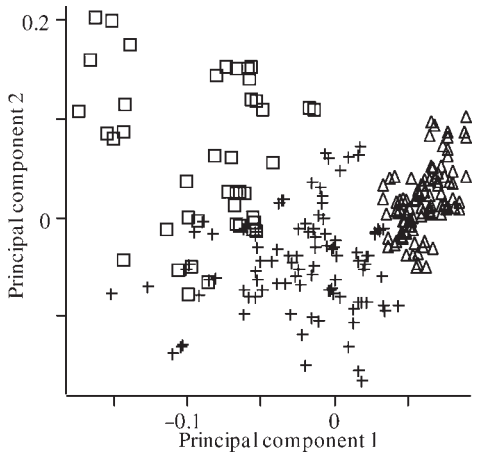

Fig. 4. PCA score plot for UPLC-MS data of the triplicate *S. marinoi* cell extracts. The exponential (triangle), the stationary (cross) and the declining (square) phase are separately labeled. The first principal component (*x*-axis) separates the exponential and the declining phase.

DISCUSSION

Using a targeted addition of nutrients and inoculation of *S. marinoi* cells, we were able to alter plankton assemblages in mesocosms. Feeding of *Calanus* spp. on these manipulated phytoplankton communities was observed. Because conditions remained fairly constant throughout the experiment, variable environmental factors like light or temperature could be excluded as main factors influencing copepod feeding. Besides the inoculated *S. marinoi*, a wide diversity of other phytoplankton species was present ensuring that copepods could select food from a complex phytoplankton species assemblage. Using a prey-specific semi-quantitative molecular assay, we were able to monitor the gut content of single *Calanus* spp. individuals *in situ* without the need for

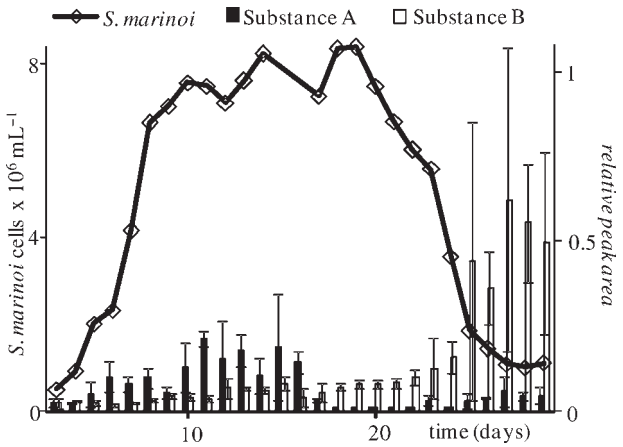

Fig. 5. Bars: Relative peak area of 259 m/z at 1.48 min (black) and 448 m/z at 4.43 min (white) in the *S. marinoi* cells as a function of culture age ($n = 3$). Integrals were normalized to extraction volume and cell number. Error bars indicate the standard deviation. Line: *S. marinoi* average cell number of triplicates as a function of time. Cell counts are from Barofsky *et al.* (Barofsky *et al.*, 2009).

excessive manipulation (Troedsson *et al.*, 2007; Nejstgaard *et al.*, 2008). The new approach allowed us to avoid lengthy bottle incubations that would influence the plankton composition and the metabolic profile of prey algae during the experiments.

Feeding on *S. marinoi* during the experiment did not directly correlate with the concentration of this alga in the seawater (Fig. 1). Instead, an increased feeding on this diatom was observed during the later stages of the experiment, when cell counts of *S. marinoi* were already decreasing. Grazing on *S. marinoi* was either suppressed during the peak of the induced algal bloom, or grazing on late bloom cells was stimulated. Since the communities in the mesocosm experiments were heterogeneous, we cannot conclude whether and to what extent the abundant alternative food was selected during the peak of the *S. marinoi* bloom. Both a general reduction of feeding activity and/or a selective ingestion of other food particles are valid explanations for the observed results.

It should be noted that only during the height of the *S. marinoi* bloom in mesocosm 2 the protist biomass was dominated by *S. marinoi*, while in mesocosm 1 and during the later phase in mesocosm 2 more abundant alternative food such as ciliates were available (Fig. 2). Ciliates and larger dinoflagellates were generally the most preferred prey for *Calanus* in previous mesocosm experiments at this site (Nejstgaard *et al.*, 1997, 2001). Results from mesocosm 1 and from the lab experiment thus make a feeding behavior according to the optimal foraging theory an unlikely explanation for the observed effects. This theory predicts that copepods would exhibit a concentration-dependent selectivity for high quality foods when food is more abundant since more time can be spent selecting the best particles (DeMott, 1995). But since the expected preferred prey (ciliates and dinoflagellates) were abundant and increasing during our experiments, such a mechanism should not be relevant. In fact, if there were no changes in the palatability of *S. marinoi*, an opposite result with a decrease in feeding rate on the diatom in the later stage would be expected. The mesocosms thus support that *S. marinoi* was a relatively more palatable food when in later growth stages.

In order to gain further insight into the underlying processes governing food selection, we set up laboratory experiments in which copepods were given a selection of a mixed diet of *R. marina* and *S. marinoi* under controlled conditions. The same *S. marinoi* isolate used in the mesocosm inoculations was used for the laboratory experiment to avoid influence of different chemical properties in between different isolates (Wichard *et al.*, 2005). As observed in the mesocosm experiments, a significantly higher proportion of *S. marinoi* in the stationary phase was ingested when compared with *S. marinoi* in exponential growth phase. Basic nutritional properties that could be relevant for food selection did not significantly change during the course of the mesocosm experiments. Published data for *S. marinoi* cultures indicate no significant differences in the C/N ratio between exponential and stationary phase (Ribalet *et al.*, 2007). Since *S. marinoi* did not appreciably change its cell size or morphology during our culturing (visual observations), an active selection mechanism based on factors other than C/N, shape and size is indicated. Also the release of polyunsaturated aldehydes from *S. marinoi* cultures seems to have no negative impact on feeding, since it occurs only during the late stationary phase where no feeding inhibition was observed (Vidoudez and Pohnert, 2008).

Copepods are well equipped with chemosensory organs to mediate prey selection (Friedman and Strickler, 1975). Different amounts of chemical signals such as defensive metabolites or specific nutrients could therefore explain the observed food selection. Selection based on such chemical factors may be based on detection of released metabolites or on the evaluation of intracellular chemical properties detected during the feeding process. Extracellular polysaccharides can indeed facilitate the selection process (vanDonk *et al.*, 1997). Malej and Harris (Malej and Harris, 1993) reported that the release of polysaccharides by diatoms, which occurs during later growth phases, leads to a decreased food uptake of copepods, a result that was later confirmed for exudates of *Phaeocystis globosa* (Dutz *et al.*, 2005). In contrast, we observed increased food uptake during late phases of an induced *S. marinoi* bloom as well as during stationary phase of a culture. In a previous study, we demonstrated that the profile of exuded metabolites (excluding saccharides) of *S. marinoi* is highly dependent on the growth phase of the algal culture (Barofsky *et al.*, 2009). Accordingly, specific blends of metabolites can be observed in the water around the algae dependent on the age of the culture. These might be exploited by copepods as indicators for their food selection (Wolfe, 2000).

To verify whether changes in the released metabolites are also reflected by the changes of metabolites found in the cells, we performed a metabolic profiling of cell extracts. Metabolites detected during this analysis correspond directly to those ingested during feeding and may be most relevant for copepod preference. Major differences in the metabolic profiles of the cells during the growth periods of a culture could be detected (Fig. 4). A first evaluation of the structural properties of the detected metabolites using mass spectra and retention times revealed that the high plasticity of chemical profiles

of the diatoms throughout bloom development is caused by more than established metabolites, such as lipids, amino acids and saccharides (Jonasdottir, 1994). We thus observe a high degree of variation of intracellular and extracellular metabolites during culturing. These changes due to aging of a culture or bloom make the cells during a late culturing phase or bloom chemically distinguishable from cells during early stages. Since these changes are apparently linked to changes in feeding behavior, they have the potential to influence plankton composition. Our results have broader implications for lab and field experiments since they indicate that there is no general palatability of an algal species but rather a high plasticity throughout a culture or bloom.

We conclude that variation in the age of a diatom bloom under field conditions has the power to significantly alter copepod feeding and thereby potentially influence plankton community structure. Besides the implications for the food web structure, the ecological reason for such a variable palatability leaves much room for speculation. For example, diatoms may invest more resources in chemical defense during early growth phases. We cannot fully exclude other factors responsible for selection, like a switch in feeding behavior or grazing according to the optimal foraging theory even if known indicators for these behavioral responses were not observed in our experiments. Alternatively, they may contain more nutritionally valuable metabolites in later growth phases. To answer such questions on the mechanism of chemically mediated interactions, it will be a major challenge to identify the metabolites or metabolic blends involved in the predator–prey interaction. Future research will require structure elucidation and testing in bioassays, a task beyond the scope of this study. The qPCR method that allows the monitoring of ingested food items will provide an adequate tool for analyses of the gut content facilitating a bioassay guided fractionation of the active metabolites.

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