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The taste of diatoms: the role of diatom growth phase characteristics and associated bacteria for benthic copepod grazing

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ABSTRACT: The interactions between primary producers and their consumers are of particular interest for the overall functioning of marine ecosystems. The biochemical composition of the organisms involved affects the efficiency of energy transfer in marine food webs. In addition to top-down control by grazers, bottom-up control of these interactions by primary producers and associated bacteria has recently received more attention. Planktonic copepods selectively feed on older diatom cells, a behaviour regulated by changes in exometabolites around diatoms. To test whether this also applies to benthic copepods, *Seminavis robusta* cells in lag, exponential and stationary growth phases were biochemically screened, and the diversity of associated bacteria was assessed. The diatoms were subsequently ^{13}C prelabelled and offered to the harpacticoid copepod *Microarthridion littorale* in a grazing experiment. Harpacticoid copepods incorporated more carbon from younger diatom cells in the lag growth phase, which might be based on (1) biochemical differences of diatom cells in different growth phases and (2) the bacteria associated with the diatoms. The younger diatom cells were characterised by a higher C:N ratio and more extracellular polymer secretions but a lower fatty acid content. The bacterial community on these cells differed from those on cells in the later growth phases. Our results thus suggest that the feeding strategies of benthic harpacticoid copepods differ from those of calanoid copepods. This outcome can be explained by the tight contact between benthic copepods and the typical carbon and bacteria-rich biofilm on sediments.

KEY WORDS: Diatoms · Grazing · Harpacticoid copepods

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INTRODUCTION

Copepods play a pivotal role in marine food webs, both as consumers of primary production and as food source for higher trophic levels. The interactions between primary and secondary producers are of particular interest for the functioning of the marine ecosystem since the efficiency with which biomass and energy are transferred is highly variable (Brett & Goldman 1997, Micheli 1999). At this plant–animal interface, differences in the biochemical and mineral composition of organisms are most pronounced and consequently lead to differences in trophic transfer efficiencies that are among the largest in the food web. For instance, there is evidence that a poorer nutritional composition leads to enhanced feeding rates as a result of compensatory feeding (e.g. Augustin & Boersma 2006).

In estuaries, primary producers may experience tidal-driven variation in environmental conditions (e.g. light, nutrients, temperature and salinity fluxes), potentially leading to further variability in biochemi-

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cal composition and the strength of trophic interactions. Diatoms represent the main source of phototrophic primary production in the biofilms that cover intertidal sediments (Decho 2000) and constitute the main food source for benthic harpacticoid copepods grazing on these biofilms (e.g. Pace & Carman 1996, Buffan-Dubau & Carman 2000). Lubarsky et al. (2010) underlined the important role of heterotrophic bacteria in 'biostabilisation' of intertidal sediment by means of biofilms and highlighted the interactions between autotrophic and heterotrophic biofilm consortia.

In spite of the abundance of food in these environments, harpacticoid copepods are able to discriminate among diatoms based on cell shape (Wyckmans et al. 2007) and cell size (De Troch et al. 2006a). Planktonic copepods, however, have the additional ability to select diatoms based on their growth phase (Barofsky et al. 2010), which is known to be associated with marked differences in exometabolite production (Barofsky et al. 2009). The resulting complex chemical sphere around phytoplankton cells, the so-called exometabolome or phycosphere (Moore et al. 1999), might have important implications for the organisms grazing on the cells (see Barofsky et al. 2010). Since the composition of the phycosphere changes over time, it has the potential to transmit information on the physiological state of the cell to grazing copepods (Moore et al. 1999). In contrast, Jónasdóttir et al. (2011) recently found no effect of different fatty acid compositions of diatoms on egg production of planktonic copepods *Calanus finmarchicus*.

Whereas the mechanisms of the interactions between diatom cells and (copepod) grazers in the planktonic realm have received much attention (e.g. Jones & Flynn 2005, Pohnert 2005, Siuda & Dam 2010, Jónasdóttir et al. 2011), little is known regarding the interactions between diatom cells and benthic harpacticoid copepods. In contrast to planktonic copepods, which create a feeding current and have a greater potential for remote chemoreception (Moore et al. 1999), benthic copepods occur in a sediment matrix and have short antennae, both unfavourable for actively creating water currents. However, as these copepods live in close vicinity to sediment surfaces covered by biofilms consisting of diatoms and bacteria (Decho 2000), a direct influence of the metabolites released by benthic diatoms may be expected. In addition, the role of bacteria associated with diatoms in any transformation of these compounds and their consequences for the feeding of grazers on the biofilm needs clarification.

The present study aimed to document changes in the biochemical composition of exudates (mainly carbohydrates), diatom cells (pigments, lipids and carbohydrates) and associated bacterial communities among growth phases of diatom cultures and their effect on grazing by harpacticoid copepods. In planktonic communities, copepods showed a preference for diatom cells in the late stationary phase (Barofsky et al. 2010). The quantitative PCR gut content assessment used by Barofsky et al. (2010) to reveal the food uptake by the copepod *Calanus* sp. provides, however, no quantitative data on the assimilation of diatom carbon by copepods from various diatom growth phases. By means of grazing experiments with ^{13}C prelabelled diatoms, we aimed to provide quantitative data on the carbon assimilation by benthic copepods and how this changes as diatom cells reach different growth phases. Overall the aim of the study was to determine whether diatoms and their associated bacteria impose bottom-up control on marine grazers, or if different forces govern diatom-bacteria-copepod interactions in benthic and planktonic communities.

MATERIALS AND METHODS

Culture conditions and labelling technique

Cultures of the epipelic pennate diatom *Seminavis robusta* have been repeatedly shown to be a suitable food source for various harpacticoid copepods in laboratory experiments (e.g. De Troch et al. 2006a,b). In the present study, we used *S. robusta* strain 85A, which represented the F2 generation derived originally from the cross of 2 sexually compatible clones (the mating system of *S. robusta* is heterothallic) (Chepurnov et al. 2002). The strain is cryopreserved in the diatom culture collection (BCCM/DCG) of the Belgian Coordinated Collection of Micro-organisms (http://bccm.belspo.be) and can be ordered online (accession number DCG 0105). The original strains were isolated from a sample collected in November 2000 from the 'Veerse Meer', a brackish water lake in Zeeland, The Netherlands (Chepurnov et al. 2002). Cell length at the time of the experiment was 62.5 ± 2.0 µm (mean ± standard error [SE]). The cultures were grown in a climate room at 17 ± 1°C with a 12:12 h light-dark period and 25 to 50 µmol photons m^{-2} s^{-1}. The culture medium was f/2 (Guillard 1975), which was based on filtered and sterilised artificial seawater (salinity: 32, Instant Ocean® salt, Aquarium Systems). Non-

axenic cultures were started with an initial density of 50 cells cm^{-2} in 200 ml f/2 medium in tissue bottles (total bottom surface: 175 cm^2), and cell densities were counted daily under an inverted microscope (Zeiss Axiovert 40C) during 1 month to construct a growth curve and define the different growth phases (lag, exponential and stationary).

Prior to the grazing experiment, cultures were started at different points in time (i.e. to grow for 22, 16 and 5 d for the stationary, exponential and lag phase, respectively) and in a different number of bottles (i.e. 9, 6 and 3 bottles for lag, exponential, and stationary phase, respectively) to be able to harvest sufficient cells of each growth phase. Diatom cells were grown in tissue bottles (175 cm^2 surface, 200 ml f/2 medium) and labelled with the stable isotope ^{13}C by adding 5 ml of a solution with $NaH^{13}CO_3$ (sodium bicarbonate, ^{13}C, 99%, Cambridge Isotope Laboratories, 336 mg in 100 ml milliQ H_2O) per 100 ml culture medium. The labelling technique resulted in an increase in $\delta^{13}C$ in the cells from −12.2‰ (control) to 260.4‰ (lag), 8317.1‰ (exponential) and 6046.6‰ (stationary).

Grazers (harpacticoid copepods) for the present study were collected from silty sediments at the Paulina intertidal flat (SW Netherlands, 51°21′ N, 3°43′ E). Adults of the dominant harpacticoid copepod *Microarthridion littorale* (family Tachidiidae) were extracted alive from the sediment using a mixed technique of sediment decantation and application of white light to attract positive phototactic copepods. Copepods were washed 3 times and placed overnight in petri plates with 15 ml of artificial seawater (17 ± 1°C, 12:12 h light-dark cycle) to empty their guts prior to the start of the experiment.

Biochemical characterisation of diatoms

Triplicate samples were collected for biochemical characterisation of diatoms including pigments (mainly chlorophyll *a* [chl *a*] concentration), nutrients, C:N ratio and fatty acids profile by filtering 7, 4 and 3 bottles over preglown Whatman glass fibre filters (GF/F) for each replicate of the lag, exponential and stationary growth phase, respectively. The resulting filters and supernatants (for nutrients) were immediately stored frozen (−80°C for pigments and fatty acids, −20°C for the other samples). Separate bottles (3 per growth phase) were used to collect aliquots of growth medium with cells for the analysis of extracellular polymeric secretions (EPS) concentration (samples of 10 ml) and diversity of associated bacteria (samples of 1 ml for DGGE, see section 'Bacteria associated with diatoms').

Photopigments were extracted from the cells on the filters by adding 10 ml 90% acetone to the lyophilised GF/F filters at 4°C in the dark and were determined in the resulting supernatant by reverse phase liquid chromatography on a Gilson high performance liquid chromatography chain (spectrophotometric and fluorometric detection) according to a modified protocol of Wright & Jeffrey (1997).

Nutrient (NO_2^- – N, NO_3^- – N, NH_4^+ – N, PO_4^{3-} – P and SiO_2) concentrations in 15 ml of filtered growth medium were analysed using an AII automatic chain (SANplus Segmented Flow Analyser, SKALAR).

The C:N ratio of freeze-dried diatom cells in each growth phase was determined from measurements of particulate organic carbon and particulate organic nitrogen by a Flash 2000 Series organic elemental analyser (Thermo Scientific).

For each growth phase, the EPS concentration in 10 ml of growth medium with diatom cells was measured in 4 different fractions (soluble EPS, bound EPS, internal sugars [mainly storage glucans] and residual sugars) following the protocol of De Brouwer & Stal (2002) based on the phenol-sulfuric acid assay (Dubois et al. 1956). Soluble EPS was obtained by centrifuging 10 ml of diatom culture at 3500 × *g* for 15 min at room temperature. The supernatant was transferred to a centrifuge tube containing 30 ml cold ethanol (98%), and the EPS was allowed to precipitate overnight at −20°C. After centrifugation (15 min at 3500 × *g*), the EPS pellet was dried under a flow of nitrogen and subsequently resuspended in 300 µl of Milli-Q water. A volume of 200 µl was used for analysis of carbohydrates. Bound EPS was extracted by resuspending the culture pellet in 2 ml of Milli-Q water. The cell suspension was thoroughly stirred and incubated for 1 h at 30°C. After centrifugation at 3500 × *g* for 15 min, the EPS was isolated. Glucans were isolated by using the procedures developed by Myklestad et al. (1972) by adding 1 ml 0.05 M H_2SO_4 to open the diatom cells to analyse the internal sugars in the cell pellet. The suspension was mixed every 30 min for a period of 2 h. After centrifugation (3500 × *g*, 15 min), 200 µl of supernatant was used for carbohydrate analysis. For determination of the residual carbohydrates, the cell pellet was resuspended in 400 µl of Milli-Q. A volume of 200 µl of this suspension was used for analysis. This residual carbohydrate fraction mainly consisted of cellular carbohydrate (Smith & Underwood 2000). All carbohydrates were measured using the phenol-sulfuric acid assay (Dubois et al. 1956). To each 200 µl sample,

200 µl phenol (5%, w/v in Milli-Q water) and 1 ml concentrated H_2SO_4 was added. This mixture was shaken and then incubated for 35 min, and the absorbance at 486 nm was measured using a Victor^3 multilabel reader (Perkin Elmer). Glucose was used as a standard.

Hydrolysis of total lipid extracts (in triplicate) of the diatoms in each growth phase and methylation to fatty acid methyl esters (FAMEs) was achieved by a modified 1-step derivatisation method after Abdulkadir & Tsuchiya (2008). The boron trifluoride-methanol reagent was replaced by a 2.5% H_2SO_4-methanol solution since BF_3-methanol can cause artefacts or loss of polyunsaturated fatty acids (Eder 1995). The fatty acid methylnonadecanoate C19:0 (Fluka 74208) was added as an internal standard for the quantification. The FAMEs thus obtained were analysed using a Hewlet Packard 6890N gas chromatograph with a mass spectrometer (HP 5973) via a splitless injection at an injector temperature of 250°C using a HP88 column (Agilent J&W, Agilent). The FAMEs were analysed with the software MSD ChemStation (Agilent Technologies). Quantification of individual FAMEs was accomplished by linear regression of the chromatographic peak areas and corresponding known concentrations (ranging from 5 to 150 µg ml^{-1}) of external standards (Supelco #47885, Sigma-Aldrich).

Shorthand FA notations of the form A:BωX were used, where A represents the number of carbon atoms, B gives the number of double bonds, and X gives the position of the double bond closest to the terminal methyl group (Guckert et al. 1985).

Bacteria associated with diatoms

Bacterial DNA was prepared through alkaline lysis (Baele et al. 2000) from triplicate samples (1 ml each) of the diatoms in the different growth phases (3 bottles per growth phase were available). From each DNA extract, an internal 194 base pair fragment of the V_3 region of the 16S rRNA gene was amplified using the primer set 357f and 518r (Yu & Morrison 2004) (Sigma Aldrich) with a GC-clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG-3′) (Temmerman et al. 2003) coupled to the forward primer. PCR mixtures were prepared according to Temmerman et al. (2003). Starting from 1 µl of DNA template, a touchdown PCR (Van Hoorde et al. 2008) with 10 cycles of decreasing annealing temperature (0.5°C cycle^{-1} decrement, from 61 to 56°C) followed by 25 cycles of regular PCR was performed with a Bio-Rad DNA thermal cycler. Subsequent DGGE analysis using a 35 to 70% gradient and staining of the gel were done as described by Van Hoorde et al. (2008).

Digitized DGGE gels were normalized and analysed using the BioNumerics program (version 4.61, Applied Maths). Calculation of the Jeffrey's correlation coefficient and application of unweighted pair group method with arithmetic mean resulted in a dendrogram visualizing similarity between the band patterns of different growth phases.

Uptake of diatoms by copepods: experimental design

The uptake of diatom cells in a certain growth phase by copepods was tested by means of a grazing experiment with ^{13}C prelabelled diatoms (see 'Culture conditions and labelling technique'). Prior to the experiment, the ^{13}C labelled culture medium of diatoms was gently removed by suction with a glass Pasteur pipette (connected to a water jet pump) and replaced by autoclaved artificial seawater (salinity: 32) without any f/2 medium to avoid additional growth of the diatoms. During this washing process (repeated 3 times), the diatoms remained undisturbed at the bottom of the culture bottle. Diatom cells were concentrated as such and counted, and the correct amount was transferred to different treatments (see next paragraph). At the start of the experiment, triplicate samples for the copepod *Microarthridion littorale* (20 adults each) and diatoms (each growth phase) from the field were stored at −20°C for stable isotope analysis as a control for initial labelling.

The experimental design consisted of 3 simultaneous treatments with 3 replicates each: (1) copepods fed with ^{13}C labelled diatoms in lag phase, (2) copepods fed with ^{13}C labelled diatoms in exponential phase and (3) copepods fed with ^{13}C labelled diatoms in stationary phase. A total of 0.5×10^6 diatom cells per experimental unit (petri plate, 21.2 cm^2 surface, max. 15 ml volume of autoclaved artificial seawater, salinity: 32) was applied in each treatment (irrespective of the growth phase) by means of a micropipette. Per unit (replicate), 20 adult copepods were added after ~15 min to allow all diatom cells to settle and form a homogeneous layer at the bottom of the petri plate (M. De Troch pers. obs.). All experimental units were placed at random on 1 shelf in a thermostatic room (17 ± 1°C, 12:12 h light-dark regime). Diatom cells remained constant in size and morphology throughout the duration of the experiment (4 d) (authors' pers. obs.).

After 4 d, the experiment was terminated by estimating the mortality of copepods in the experimental unit and sorting them for further analysis. From each replicate, all 20 copepods were picked out using a micro needle and stored at –20°C for further total ^{13}C analysis. Afterward, they were thawed and washed 5 times in deionised water. All copepods from 1 replicate were transferred into 1 tin capsule (8 × 5 mm, Elemental Microanalysis) within 2 h (to avoid label leakage) and desiccated overnight at 60°C. For each replicate, the $\delta^{13}C$ values and copepod biomass (total carbon) were measured with a continuous flow isotope ratio mass spectrometer (Europa Integra) at the UC Davis Stable Isotope Facility (University of California, USA).

Incorporation of ^{13}C is reflected as excess (above natural abundance) ^{13}C, reported as (1) specific uptake ($\Delta\delta^{13}C = \delta^{13}C_{sample} - \delta^{13}C_{control}$, where $\delta^{13}C$ is expressed relative to Vienna Pee Dee Belemnite [VPDB]) and (2) as total uptake in µg ^{13}C per individual (Middelburg et al. 2000). The latter is calculated as the product of excess ^{13}C and individual biomass (organic carbon). Excess ^{13}C is the difference between the fraction ^{13}C of the control copepods ($F_{control}$, i.e. based on the natural signature of copepods from the field) and the fraction ^{13}C of the treatment copepods ($F_{treatment}$, i.e. based on the signature of copepods feeding on a labelled diet), where $F = {}^{13}C \div ({}^{13}C + {}^{12}C) = R \div (R + 1)$. The carbon isotope ratio (R) was derived from the measured $\delta^{13}C$ values as $R = [\delta^{13}C \div (1000 + 1)] \times R_{VPDB}$, with $R_{VPDB} = 0.0112372$ as $\delta^{13}C$ is expressed relative to VPDB.

Since the offered food sources (diatoms in different growth phases) had different initial $\Delta\delta^{13}C$ signatures (260.4‰ for lag, 8317.1‰ for exponential and 6046.6‰ for stationary), the total uptake per individual was further standardized taking into account the proportion of ^{13}C in each food source. The amount of total carbon that was taken up by copepods and expressed per individual copepod in the treatments was multiplied by the factors 71.8, 11.8 and 13.6 for the treatments with labelled diatoms in lag, exponential and stationary phase, respectively. These correction factors were derived from the atom % ^{13}C excess in each growth phase, i.e. on average, 1.4, 9.4 and 7.3% ^{13}C for lag, exponential and stationary phase, respectively.

Data analysis

Differences in uptake among treatments and changes in biochemical composition of diatoms in different growth phases (sampled in independent bottles) were tested by means of 1-way analyses of variance (ANOVA) with Statistica 6.0 software (StatSoft 2001). *A posteriori* comparisons were carried out with the Tukey honestly significant difference test using 95% confidence limits. Prior to all ANOVAs, the Cochran's *C*-test was used to check the assumption of homoscedasticity.

RESULTS

The 3 phases in the growth curve of *Seminavis robusta* (Fig. 1) were defined as lag phase (0 to 200 h, Day 0 to 8), exponential phase (200 to 500 h, Day 9 to 20) and stationary phase (from 500 h onwards). Based on this growth curve, diatoms were collected from independent culture bottles after 5 (lag phase), 15 (exponential phase) and 22 d (stationary phase) for biochemical characterisation of the cells in each growth phase.

Biochemical changes during the growth of diatoms

Parallel with the increasing number of diatom cells as growth progressed, chl a concentrations changed considerably, ranging from 1.7 µg l^{-1} in the lag phase to 4.7 µg l^{-1} in the exponential phase to 157.9 µg l^{-1} in the stationary phase. A standardisation to the number of cells showed a decreasing cellular chl *a* concentration with increasing cell density in the cultures: the lowest chl *a* content (0.9×10^{-5} µg $cell^{-1}$) in the stationary phase as cells became older versus 2.7×10^{-5} µg $cell^{-1}$ and 1.6×10^{-5} µg $cell^{-1}$ in the lag and exponential phase, respectively.

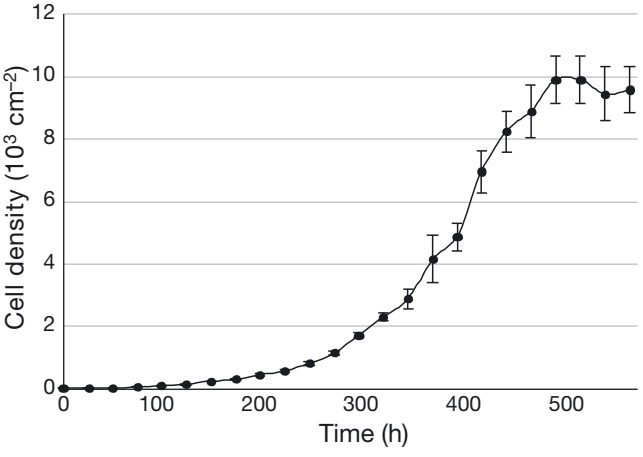

Fig. 1. *Seminavis robusta*. Average growth curve (mean cell density ± SE, n = 3)

Monitoring of the nutrient use by diatoms during their growth (Fig. 2) showed that there were some major changes in the composition of the f/2 medium. There was a significant decrease in the amount of silica (Si) (Fig. 2a) diminishing from 144.6 ± 0.4 µmol l^{-1} in the initial f/2 medium to 99.2 ± 1.3 µmol l^{-1} (lag), 80.9 ± 9.4 µmol l^{-1} (exponential) and 69.9 ± 7.7 µmol l^{-1} (stationary) (mean values ± SE, 1-way ANOVA, $p = 0.004$), implying that half of the initial amount of silica in the f/2 medium was already used by the time the cells reached the stationary phase. Most of the nitrite in the f/2 medium (Fig. 2b) was used for growth as the concentration dropped from 2.02 ± 0.02 µmol l^{-1} to below the detection limit in all growth phases. Nitrate (Fig. 2c) and phosphate (Fig. 2d) concentrations, in contrast showed no significant changes during diatom growth.

The C:N ratio of diatom cells was significantly higher in the initial lag phase (10.7 ± 0.1) than in the later growth phases (exponential phase: 8.8 ± 0.1, stationary phase: 8.7 ± 0.2) (mean values ± SE, 1-way ANOVA, $p < 0.001$, post-hoc Tukey test, $p < 0.001$). In contrast, the incorporation of ^{13}C from the added labelled sodium bicarbonate showed the lowest atom % ^{13}C in the initial lag phase: 1.4% (lag phase), 9.4% (exponential phase) and 7.3% (stationary phase).

The highest concentration of EPS (mean values ± SE) was recorded for the lag phase, and especially the soluble EPS ($6.2 \times 10^{-8} \pm 1.2 \times 10^{-8}$ g $cell^{-1}$), glucans ($3.3 \times 10^{-8} \pm 0.2 \times 10^{-8}$ g $cell^{-1}$) and residual carbohydrates ($3.7 \times 10^{-8} \pm 0.7 \times 10^{-8}$ g $cell^{-1}$) showed higher levels than in the later growth phases (Fig. 3). In the stationary phase, the amount of EPS decreased sharply, with total EPS reaching the average value of $1.4 \times 10^{-9} \pm 0.2 \times 10^{-9}$ g $cell^{-1}$.

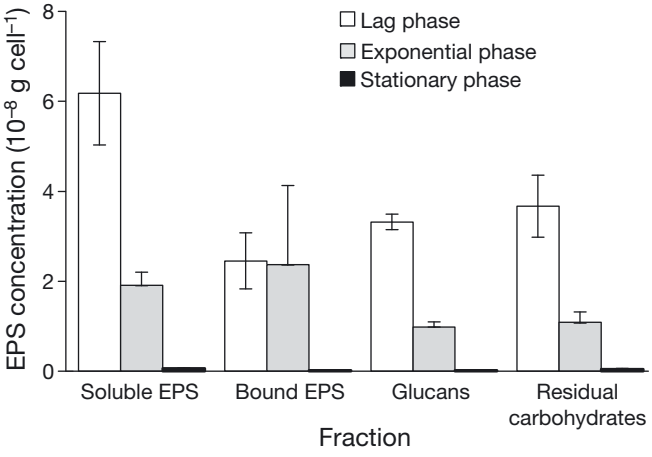

Fig. 3. Concentrations of exopolymer secretion (EPS) fractions in different growth phases; mean values ± SE

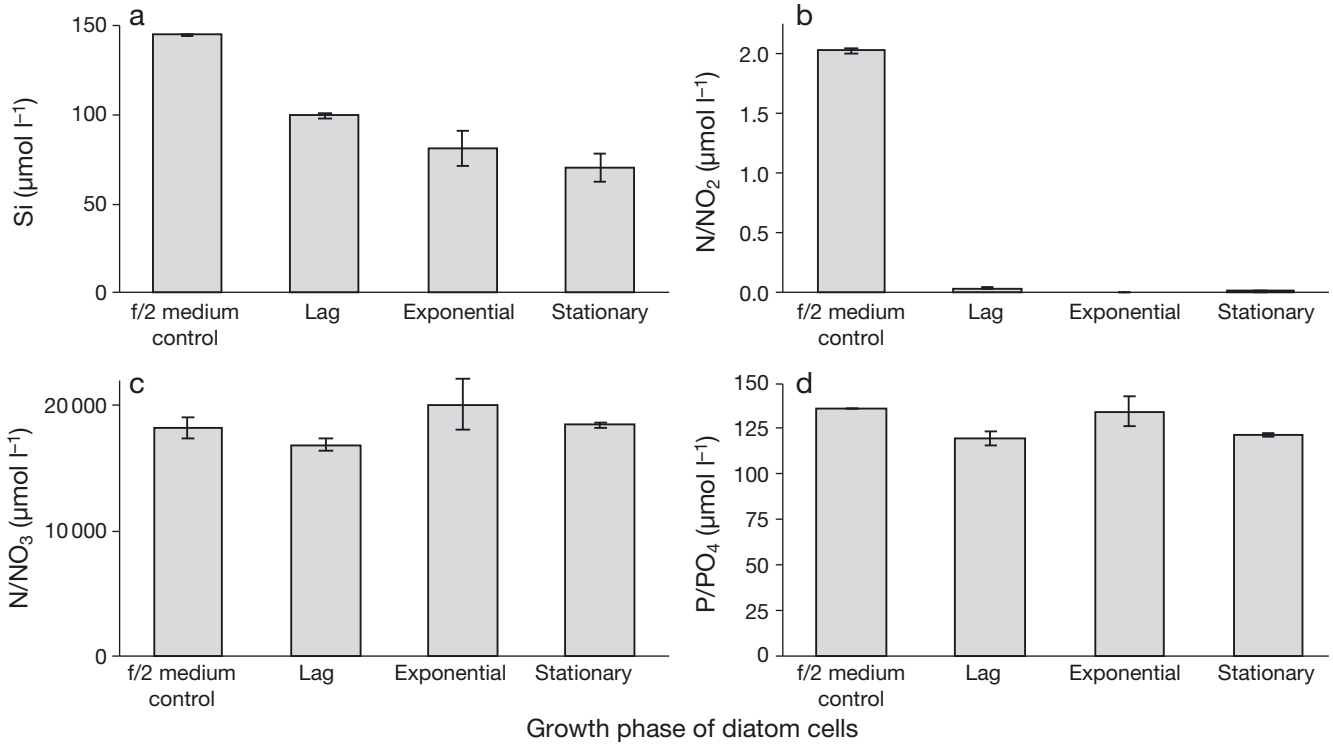

Fig. 2. Nutrient concentrations in the f/2 growth medium and in different growth phases: (a) silica, (b) nitrite, (c) nitrate and (d) phosphate; mean values (in µmol l^{-1}) ± SE

Fatty acid composition in the different growth phases differed in terms of composition, with a more diverse fatty acid composition in the stationary phase (Table 1). The polyunsaturated fatty acid docosahexaenoic acid (DHA, 22:6ω3) was only found in cells in the stationary phase at an average concentration of 19.3 ± 3.8 ng ml^{-1} (mean value ± SE). In addition, the odd-branched fatty acids (15:0 and 17:0) that are typical for bacteria were only detected for the older diatom cells. The relative contribution of the fatty acids 16:0, 16:1ω7c and 20:5ω3 (EPA) increased as the cells got older (in the stationary phase). The fatty acid 18:1ω9t, in contrast, decreased toward the stationary phase.

Bacterial communities on diatoms of different age

Overall, there was a high similarity among the replicates of the different growth phases (Jeffrey's Similarity > 90%) (Fig. 4), although they originated from different bottles. The bacterial community on the diatom cells in the initial lag growth phase clustered separately from the bacterial communities

Table 1. Mean absolute (ng ml^{-1} ± SE) and relative (parentheses; %) fatty acid concentrations in the different growth phases. t: trans; c: cis. Empty cells: absence of FA or below detection limit

	Lag phase	Exponential phase	Stationary phase
12:0	2.20 ± 1.82 (3)	1.81 ± 1.19 (2)	4.57 ± 1.33 (0.6)
14:0	1.36 ± 0.56 (1.9)	1.61 ± 0.72 (1.7)	35.39 ± 16.47 (4.5)
14:1ω5	1.07 ± 0.12 (1.5)	0.19 ± 0.19 (0.2)	1.40 ± 1.40 (0.2)
15:0			0.68 ± 0.68 (0.1)
16:0	13.15 ± 3.45 (18)	16.32 ± 7.64 (17.7)	224.75 ± 135.22 (28.7)
16:1ω7t	4.77 ± 1.23 (6.5)	5.08 ± 2.23 (5.5)	4.35 ± 0.00 (0.6)
16:1ω7c	20.52 ± 4.34 (28.1)	29.28 ± 12.26 (31.8)	327.54 ± 198.32 (41.8)
17:0			1.79 ± 0.00 (0.2)
18:0	2.03 ± 0.73 (2.8)	2.37 ± 1.17 (2.6)	3.11 ± 1.30 (0.4)
18:1ω9t	21.48 ± 4.32 (29.4)	22.68 ± 9.90 (24.6)	24.07 ± 9.32 (3.1)
18:1ω9c	3.21 ± 0.98 (4.4)	7.19 ± 3.10 (7.8)	13.36 ± 4.50 (1.7)
18:3ω6	1.62 ± 0.32 (2.2)	3.24 ± 1.19 (3.5)	21.45 ± 8.11 (2.7)
18:3ω3		0.18 ± 0.00 (0.2)	29.35 ± 25.93 (3.7)
20:5ω3 (EPA)	1.58 ± 0.25 (2.2)	2.23 ± 0.77 (2.4)	72.69 ± 63.86 (9.3)
C22:6ω3 (DHA)			19.34 ± 3.79 (2.5)

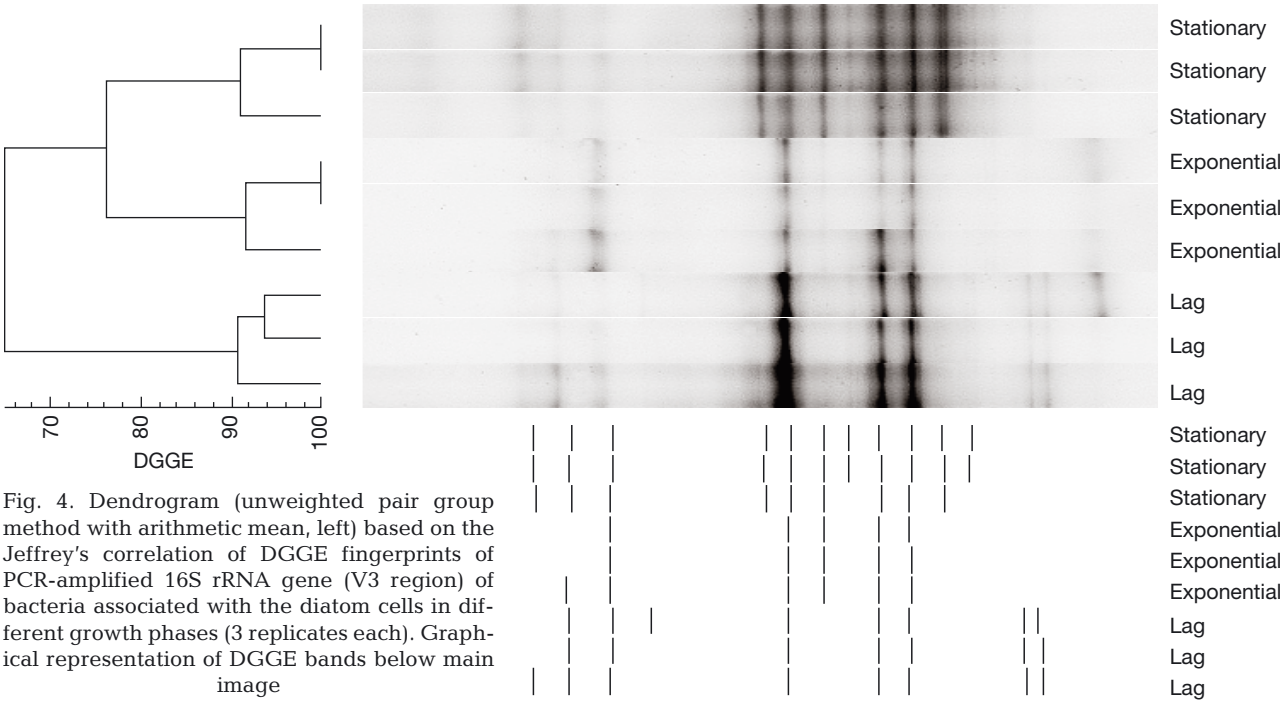

Fig. 4. Dendrogram (unweighted pair group method with arithmetic mean, left) based on the Jeffrey's correlation of DGGE fingerprints of PCR-amplified 16S rRNA gene (V3 region) of bacteria associated with the diatom cells in different growth phases (3 replicates each). Graphical representation of DGGE bands below main image

on the older diatom cells (Jeffrey's Similarity = 65%). The bacterial communities on the cells in the exponential and stationary phases showed a somewhat higher similarity (Jeffrey's Similarity = 75%). Nevertheless, diatoms cells in the stationary growth phase harboured a more diverse bacterial community, as suggested by the higher number of bands on the DGGE gel. Altogether, this indicates that each growth phase was characterised by a typical bacterial community that differed from that on cells in other growing phases (similarity max. 75% and even lower when other similarity indices were applied).

Diatom grazing by the harpacticoid copepod *Microarthridion littorale*

The average mortality of copepods after 4 d was low (1 ± 1 ind. on average ± SD) and did not differ among the treatments.

Specific uptake ($\Delta\delta^{13}C$; Fig. 5) of *Seminavis robusta* cells by *Microarthridion littorale* was highest when copepods fed on lag phase diatom cells. Since the diatoms in the lag phase were far less enriched in ^{13}C than those in the other growth phases, a further standardisation resulted in an even higher total uptake per individual copepod (right y-axis of Fig. 5) in the lag phase in comparison to the other growth phases. This total uptake (µg ^{13}C per ind.) was up to 7- and 18-fold higher in the lag phase than in the exponential and stationary phase, respectively.

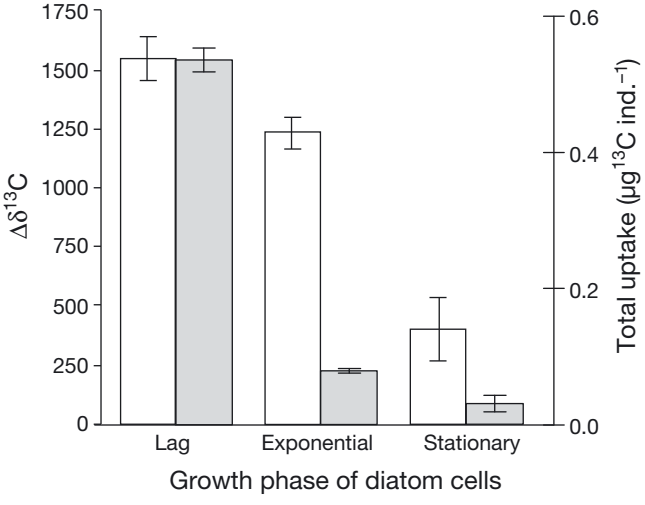

Fig. 5. Uptake of diatom cells in different growth phases by the copepod *Microarthridion littorale* expressed as (1) specific uptake ($\Delta\delta^{13}C$, white columns, plotted on left y-axis) and (2) total uptake per individual (grey columns, plotted on right *y*-axis); mean values ± SE

DISCUSSION

Although some harpacticoid copepod species are known for their ability to be highly selective in their food uptake (e.g. Azovsky et al. 2005, De Troch et al. 2006a), the present study is the first to show that they feed differently on diatom cells in different growth phases. Especially for benthic animals like harpacticoid copepods that search for their food in or on the sediment, this is a remarkable finding as a variety of potential food sources are available in natural conditions. This conclusion is not drawn from a selectivity experiment because no combined treatments were applied in the present study. Although the application of combined treatments, e.g. combining 2 growth phases with one of them being ^{13}C enriched, would be the ideal approach to test for selective feeding (e.g. De Troch et al. 2006a,b, Wyckmans et al. 2007), mixed treatments were not included because exometabolites produced by diatom cells of different age would be mixed in the experimental units and interfere with the feeding process. Instead, the use of stable isotopes (^{13}C) allowed us to estimate the amount of ^{13}C that copepods could incorporate from diatom cells in the different growth phases. This tracer technique is widely used in trophic ecology (e.g. Boecklen et al. 2011). However, a disadvantage of this technique is that the prelabelled growth medium of the diatoms needed to be removed prior to the start of the experiment since copepods could consume the ^{13}C directly from the medium. This removal, by suction with a glass pipette, could have caused a small impact on the bacteria associated with diatoms. In addition, external EPS could have been partly diluted because of this replacement of growth medium with artificial seawater (see further discussion below). In spite of these shortcomings, the uptake of ^{13}C labelled cells by harpacticoid copepods demonstrated in a timeframe of 4 d that harpacticoid copepods incorporate more diatom carbon from cells in a culture's lag phase than from cells in cultures at a later phase, with a minor variance among the replicates. Moreover, the experiment was repeated and the same outcome was found (data not shown).

The experimental set-up avoided any concentration-dependent uptake since all treatments contained the same amount of cells, independent of the growth phase. This allowed specific tests for the effect of biochemical differences of cells on the grazing of harpacticoid copepods. Therefore, the optimal foraging theory, which states that organisms would exhibit density-dependent uptake of high quality foods when food is more abundant since more time

can be spent searching for the best particles (DeMott 1995), can be excluded here as potential explanation for the grazing pattern observed. In the present study, there was no option for the grazers to search for the best particles since treatments contained only a single cell type (specific growth phase). However, to reach the same concentrations, cells had to be diluted, and consequently, this may have impacted their exudates and associated bacteria in the experimental units.

In the recent study of Barofsky et al. (2010), other potential food sources (ciliates and dinoflagellates) were present in the mesocosm, but these could not mask the preferences of the planktonic calanoid copepod *Calanus* for cells of the planktonic diatom *Skeletonema marinoi* in the late stationary phase. However, since Barofsky et al. (2010) measured only diatom DNA, there was no quantification of the role of other food sources. The higher uptake of older cells by planktonic copepods is in contrast with the higher uptake of young cells (early lag growth phase) of the epipelic diatom *Seminavis robusta* by the benthic harpacticoid copepod *Microarthridion littorale* as found in the present study.

This higher uptake of young cells may be based on differences in EPS levels. Typically more EPS is produced during later nutrient-limited growth phases; for phytoplankton (planktonic diatoms) this is reported as an 'overflow' of energy from photosynthesis (Malej & Harris 1993). Subsequently, these higher EPS levels in the stationary phase inhibit planktonic copepods grazing on the diatoms (Malej & Harris 1993). In the present study, we found the opposite pattern as the benthic diatom cells initially invested in more EPS production, and the EPS level decreased gradually as the diatom culture grew. We found not only that soluble EPS was higher in the lag phase but also internal sugar levels (storage and residual glucans) were significantly higher in younger cells. It is possible that the EPS produced in the later growth phases was taken up by associated heterotrophic bacteria (e.g. Evrard et al. 2008, Bellinger et al. 2009). In spite of the fact that the diatom cultures used in this experiment were non-axenic, diatoms were grown in autoclaved artificial seawater, and there was a minimum of bacterial growth observed. In addition, we expect mainly diatom associated bacteria to be present in the experimental units. Uptake of any bacteria by the copepods would then occur rather indirectly because of their grazing on diatoms. Consequently, any potential loss of ^{13}C label into EPS would be used by diatom associated bacteria and subsequently by grazing copepods. Therefore, this EPS production and transfer to bacteria would not mean a real loss of carbon uptake to the next trophic level.

Previous studies stated that extracellular polysaccharides can indeed facilitate the grazing selection by e.g. zooplankters *Daphnia* sp. (Van Donk et al. 1997). According to Malej & Harris (1993) and Dutz et al. (2005), the release of polysaccharide exudates by diatoms negatively affects the food uptake by planktonic copepods. In contrast, the benthic harpacticoid copepod *Microarthridion littorale* showed a higher uptake of diatom cells with a high EPS content. The test copepod used in the present study was collected from intertidal silty sediments that are typically covered by diatom biofilms (M. De Troch pers. obs.) with a high EPS content and therefore may be well adapted to high amounts of EPS. However, EPS concentrations (especially the soluble EPS fraction) could have been partly diluted by the replacement of the initial growth medium (with ^{13}C) with new artificial seawater (without ^{13}C). This laboratory experiment was conducted in petri plates without any sediment so that the diatom cells could form a biofilm on the plastic bottom of the experimental unit. We found previously (De Troch et al. 2006b) that 2 common intertidal harpacticoid species collected from the same study site showed higher diatom uptake in units without sediment than in the ones with various sediment types. Therefore, we can state that in the present experimental set-up, the grazing copepods had maximum access to the diatom biofilm and were in close contact with its exudates in view of the small volume of water (15 ml of artificial seawater) that was used. Consequently, the response of the harpacticoid copepods reported in the present study is likely to be weaker in the field when sediment is present. The number of diatoms applied in the experimental units (0.5×10^6 diatom cells per experimental unit) was sufficient for 20 copepods, as they could not finish the cells after 4 d. However, De Troch et al. (2007) showed that grazing of harpacticoids is diatom density-dependent. The diatom density applied in the present study ranks among the lowest amount of cells applied in the experiments of De Troch et al. (2007), indicating that higher diatom densities could have yielded higher uptake. In terms of copepod densities in the experimental units, 20 individuals in a petri plate with a 21.2 cm^2 surface does not lead to competition for food. The uptake of diatoms by grazing copepods can, however, depend on the grazers' densities, with a lower uptake of diatoms cells in cases with more grazers present (e.g. 20 vs. 80 copepods in the same experimental unit) (De Troch et al. 2005).

In terms of bacterial communities that were associated with the diatom cells, the initial lag phase also differed significantly from the later growth phases. Although the number of bands on a DGGE gel should be interpreted with caution as the number of bands does not always show a 1:1 correspondence to the number of bacterial operational taxonomical units, the cells in the later growth phase were characterised by a more diverse bacterial community based on the number of bands. The cells in the lag phases, in contrast, yielded more intense bands on the DGGE gel. This can point at higher concentrations of certain bacterial strains, and the diversity was also slightly higher than the bacterial community associated with the cells in the exponential phase. Since these experiments were conducted in autoclaved seawater, there was no additional source of bacteria present in the culture flasks. Consequently, all bacterial strains must have been associated with the diatoms throughout the incubation but only increased to considerable densities in a particular growth phase, resulting in the different DGGE profiles. Unfortunately, absolute densities of associated bacteria were not estimated, but typically, we saw more bacteria in older cell cultures, often clinging to each other, forming small packages (M. De Troch pers. obs.). Previous research showed that bacteria are an important food source for harpacticoid copepods (e.g. Rieper 1978, Decho & Fleeger 1988) and that some harpacticoids are highly selective for certain bacteria (Rieper 1982). Therefore, our findings in the present study underline the role of associated bacteria for the uptake of diatoms by copepods. In the present study, we did not investigate the uptake of bacteria by copepods, but the data showed a clear difference in bacteria associated with diatoms. The bacteria associated with diatoms are merely ranked as one of the possible explanations for the differential uptake of cells by copepods.

Sapp et al. (2007) reported strong shifts in bacterial communities associated with different diatom species during cultivation of the diatom cells. Similarly, Mayali et al. (2011) linked the temporal succession of different bacterial colonizers on dinoflagellates to algal bloom dynamics. Therefore, the clear differences in the bacterial communities in the DGGE analysis of the present study are not surprising. It is unclear, however, how this bacteria-diatom linkage (the so-called phycosphere) (Bell & Mitchell 1972) determines the grazing activity of the harpacticoid copepod species tested. Since no bacteria were identified by sequencing DGGE bands in the present study (but see De Troch et al. 2010 for bacteria associated with *Seminavis robusta*), it is impossible to point at certain bacteria as a discriminating factor.

Although the older diatom culture contained more fatty acids, including DHA, *Microarthridion littorale* showed a higher uptake and ^{13}C incorporation of diatom cells with a high C:N ratio, a lot of EPS and a specific bacterial community. In the present study, these factors, separately or in combination, seem to be strong triggers for grazing harpacticoid copepods on a particular epipelic diatom cell. However, alternative explanations are possible, e.g. biochemical differences in secondary metabolites (e.g. Pohnert 2007) instead of primary metabolites should not be neglected.

Although we also found that the late culturing phase is chemically distinguishable, at least in terms of fatty acid composition, from cells in the early growth stages (see also Barofsky et al. 2010), there are major differences from the latter study. First, the benthic harpacticoid copepods consumed more young diatom cells, while the planktonic copepods tested by Barofsky et al. (2010) preferred the older diatom cells. Barofsky et al. (2010) used quantitative PCR gut content assessment to reveal the food uptake by the copepod *Calanus* sp. However, the interpretation of their data is mainly based on the presence of diatom-DNA and as such can only provide evidence for selection of diatoms when the DNA of all potential food items is investigated in the gut and the copepods' feeding environment (which was not the case). The use of stable isotope tracers in the present study gives a more profound analysis of the amount of carbon that copepods get from diatoms in a particular growth phase; however, no information on selectivity could be gained from the present experimental design.

In our study, the high C:N ratio of diatom cells in the lag growth phase might support the higher uptake of young cells by harpacticoids, while Barofsky et al. (2010) excluded an active selection mechanism based on C:N ratio of the diatom *Skeletonema marinoi*. Published data for *S. marinoi* cultures indicate no significant differences in the C:N ratio between exponential and stationary phase (Ribalet et al. 2007). Moreover, the early investment in extracellular carbon production (i.e. EPS) may be linked to the benthic lifestyle of *Seminavis robusta* compared to planktonic diatoms. In the later growth phases, EPS produced by the diatoms may be taken up in the bacterial communities associated with the diatom cells, explaining the lower EPS levels in the older cells as found in the present study. It remains to be shown, however, to what extent this applies to other species.

Taken together, the results of the present study indicate that the feeding strategies of benthic harpacticoid copepods may differ from those of calanoid copepods and rely on the presence of a carbon and bacteria-rich biofilm matrix as well as on the diatoms themselves. While in the pelagic realm, concentrations of organic substrates and inorganic nutrients are generally very low, requiring physiological adaptations of primary producers and bacteria for efficient uptake and utilization of substrates as well as for long-term survival under carbon and energy limitation. This consequently leads to other effects on the next trophic level (e.g. Anderson et al. 2011).

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