Addendum I

Effect of food preservation on the grazing behaviour and on the gut flora of the harpacticoid copepod *Paramphiascella fulvofasciata*

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ABSTRACT

Harpacticoids owe their 'reputation' as primary consumers in aquatic food webs to their substantial grazing on diatoms, thus assuring an efficient energy flow to higher trophic levels. Due to the complex feeding behaviour of harpacticoids, the nature and dynamics of diatom-harpacticoid trophic interactions remain poorly understood. In addition, there is a growing interest from aquaculture industry in mass-culturing harpacticoids with algal foods but the labour costs of maintaining algal stock cultures are high. This study focuses on the palatability of preserved diatoms for copepods and considers the possible role of bacterial mediated effects on diatom food uptake.

The grazing of *Paramphiascella fulvofasciata* on a preserved freeze-dried diatom diet was tested and compared to the grazing on fresh cells. *P. fulvofasciata* assimilated the preserved diet, but assimilation of fresh cells was higher. When both cell types were mixed, no selective feeding was observed. Community fingerprinting of the bacteria associated with diatoms and fecal pellets suggests that the copepod gut flora was modified depending the food source. Furthermore, the results suggest that the egestion of gut bacteria enriches the microenvironment and this can have an additional influence on the feeding behaviour of the copepod.

Experimental research using preserved foods must take into account that copepod grazing assimilations of fresh foods are likely to be significantly higher. Yet, the stated high assimilation of the mixed diet, encourages further exploration of the application of preserved balanced foods for harpacticoid mass-culturing.

KEY WORDS: Harpacticoida, selective feeding, preserved diatoms, fecal pellet, bacterial community composition

INTRODUCTION

Meiobenthic harpacticoid copepods (Crustacea) are distributed worldwide and play a pivotal role in marine benthic food webs. Harpacticoids can feed on a wide spectrum of food items (Hicks & Coull 1983),

but their trophic link with diatoms has received most attention (e.g. Decho 1986, Montagna et al. 1995). Based on their grazing on microalgae, they are assigned the position of 'primary consumers', and the meiobenthos-algae link is considered essential for the energy flow at the basis of marine food webs (Buffan-Dubau & Carman 2000). Harpacticoids showed a positive response, as expressed by their densities and grazing rates, to microphytobenthos availability, validating the dependent relationship with its autotrophic food source (Montagna et al. 1995). Harpacticoids graze on living cells, but whether and to what extent they also utilize and participate in the recycling of dead diatoms, e.g. after the natural decline of a diatom bloom, remains unknown.

As primary consumers, harpacticoid copepods transfer primary production to higher trophic levels (Coull 1990). Copepods are important prey for a variety of other invertebrates and for juvenile or small bottom-dwelling fish (Gee 1989, Tsubaki & Kato 2009). Despite their clear significance as a food source in natural environments, utilization of harpacticoid copepods in aquaculture is limited. The superior nutritional value of copepods, mainly ascribed to their natural essential fatty acids content (McEvoy et al. 1998, Evjemo et al. 2003), renders copepods a promising live feed (Stottrup 2000). Fish cultures are typically maintained on nutritionally enriched live feeds, such as rotifers or *Artemia* (Kraul et al. 1992, Hanaee et al. 2005, Kotani et al. 2009), but supplemental feeding on copepods has shown to increase fish production yields (Heath & Moore 1997, Olivotto et al. 2008).

However, limited effort has hitherto been devoted to the development of intensive harpacticoid cultures for aquaculture applications due to their benthic life style, diverse feeding habits and complex life history. While most harpacticoid species were cultured for experimental purposes, generally using diatoms as food (Matias-Peralta et al. 2005, De Troch et al. 2006), successful attempts to scale up harpacticoid cultivation (e.g. Sun & Fleeger 1995, Stottrup & Norsker 1997, Rhodes 2003) resulted from trial-and-error application of artificial food sources (shrimp pellets, a lettuce/*Mytilus* paste, vegetable juices, yeast, fish flakes) and eventually cultivated algae. These studies illustrate the need for detailed information on the feeding requirements of harpacticoids and stress the importance of ecological research on feeding. Moreover, harpacticoid mass-rearing requires optimisation to reduce economic costs. One of the main obstacles is the need for a large-scale algal/diatom production with a sufficient supply and low labour costs. Preserving the algal food stocks through lyophilisation could partially circumvent these difficulties and would minimize the risk of contaminating the algal cultures.

The implications of preservation for feeding selectivity and efficiency remain unknown. In general, preservation (drying, freezing and freeze-drying) of microalgae is associated with a reduction of food quality but the extent of quality loss and its consequences for aquatic herbivores is still controversial (Albentosa et al. 1997, Dobberfuhl & Elser 1999). Preservation processes can modify cell morphology, the external epiflora and/or the presence of exudates and the biochemical content of cells. Considering the discriminative abilities of copepods (Rieper 1982, Cowles et al. 1988, DeMott 1988), even a minor modification of the food source can have an impact on their feeding behaviour. Acquiring knowledge about these effects on harpacticoid feeding is relevant for assessing the functional role of harpacticoids in benthic food webs, i.e. the transfer of primary production by harpacticoids, which could differ at and during diatom bloom conditions.

The importance of other potential food sources such as fecal material and associated bacteria remains underexplored. Harpacticoid copepods have the ability to discriminate between bacterial strains (Rieper 1982) and selectively assimilate a microbial diet (Decho & Castenholz 1986). Additionally, previous studies indicated that fecal pellets and associated bacterial flora may play a role in their feeding ecology. An increase in daily fecal pellet production after the removal of fecal pellets from the microcosms is an indication that harpacticoids need fecal pellets in their vicinity (De Troch et al. 2009). As fecal pellets were not actively fragmented by the copepods, attached bacteria rather than fecal pellet content may have accounted for the trophic value of the fecal pellets (De Troch et al. 2009, 2010).

This study aimed to assess the suitability of lyophilized diatom cells as compared to living cells as a food source for the harpacticoid copepod *Paramphiascella fulvofasciata*. In view of the above-mentioned nutritional links between diatoms, fecal pellets, bacteria and copepods (De Troch et al. 2005), changes in the microbial flora on the fecal pellets produced after feeding on fresh and freeze-dried cells were analysed.

MATERIALS AND METHODS

Laboratory stock cultures

The copepod species *Paramphiascella fulvofasciata* (family Miraciidae) was cultured as described by De Troch *et al.* (2005). Laboratory stock cultures were maintained in glass beakers with artificial seawater (ca. 32 psu, Instant Ocean® salt, Aquarium Systems, France) and were regularly provided with fresh benthic diatoms, the epipelic pennate species *Navicula phyllepta* and *Seminavis robusta*. The cultures were incubated at 17 ± 1 °C under a 12:12h light:dark regime with 25-50 µmol photons m^{-2} s^{-2}. Copepod specimens from the cultures reached up to 0.83 mm in adult size (body length) and a mean carbon content of 1.3 ± 0.2 µg C (including gut content).

For the experiment, the benthic diatom *Seminavis robusta* strain 84B (diatom culture collection of the Laboratory for Protistology and Aquatic Ecology, Ghent University) was grown as a food source for *P. fulvofasciata*. Diatom cells were grown in cell tissue culture flasks with f/2 culture medium (Guillard 1975) based on autoclaved artificial seawater (32 psu) and under identical light and temperature conditions as the copepod stock cultures. At the beginning of the experiment, the cells measured 32.9 ± 0.3 µm (mean ± SE) in length.

To trace food uptake, diatoms were grown in a ^{13}C-enriched f/2 medium by adding $NaH^{13}CO_3$ as described by De Troch *et al.* (2005). At the start of the experiment, the growth medium was replaced by artificial seawater (ASW) and the density of the diatom cells was estimated under an inverted light microscope.

In addition, a portion of the fresh unlabeled and labeled diatom cultures, henceforth annotated as F and F* respectively, were lyophilized. The resulting freeze-dried cells, henceforth designated as D and D* respectively, were used as a second type of food source. To remove salt crystals resulting from the ASW, the cells were washed after lyophilization with MilliQ water over a GF/F Whatman filter (pore size 0.7 µm) and briefly freeze-dried again.

The labeling technique resulted in an increase in $\delta^{13}C$ value from -17.46 ‰ ± 0.07 ‰ (mean ± SE) to 7720.98 ‰ ± 84.73 ‰ for the fresh cells and from -2.51 ‰ ± 0.21 ‰ to 8148.57 ‰ ± 19.81 ‰ for the freeze-dried cells (see further standardization of the data).

Experimental design

To determine the effect of diatom preservation on the feeding behaviour of *P. fulvofasciata*, a grazing experiment was conducted in small petri dishes (polystyrene, surface area = 21.2 cm^2). The microcosms were provided with 4 different *S. robusta* diets: labeled fresh cells (treatment F*), labeled freeze-dried cells (treatment D*), a 1:1 mix of labeled fresh cells and unlabelled freeze-dried cells (F*D) and a 1:1 mix of unlabelled fresh cells and labeled freeze-dried cells (FD*).

For each of the above treatments, 3 diatom cell densities were applied: 0.5 x 10^6 cells petri $dish^{-1}$ (low density, 'l'), 1 x 10^6 cells petri $dish^{-1}$ (medium density, 'm') and 2 x 10^6 cells petri $dish^{-1}$ (high density, 'h'), resulting in a total of 12 treatments. Each food combination (diatom type x diatom density), was

replicated 5 times. All replicates within each concentration level were standardized with respect to biomass e.g. the mixed diets contain equal biomass as the single-food diets.

After settlement of the diatom cells on the microcosm bottom surface, 20 adult specimens of *P. fulvofasciata* were collected from the laboratory stock cultures, washed multiple times in artificial seawater and starved overnight were added to the microcosms. This number of specimens guarantees at least 15 µg C copepod biomass, which is well above the detection limit of carbon isotopic measurements.

The experimental units were placed randomly on a shelf under identical temperature and light conditions as described for the stock cultures of copepods and diatoms. The units were left undisturbed for 5 days. The experiment was terminated by freezing the experimental units at -20 °C which were subsequently stored, until further processing. Prior to freezing, the mortality of the copepods was assessed in each microcosm and was found to be limited to 5.2 ± 0.3 % in all treatments.

Fecal pellets

Before freezing the experimental units, fecal pellets produced by the copepods during the 5-day period were collected for analysis of the associated bacteria.

For each food combination, fecal pellets were hand-picked, rinsed in autoclaved and filtered artificial seawater and collected in an eppendorf tube. Fecal pellet sample size was standardized by putting in 100 fecal pellets per tube. Because some microcosms contained less than 100 pellets, 2 fecal pellet samples of each food combination treatment were prepared by pooling fecal pellets from 2 or 3 replicate microcosms. The fecal samples were stored at -20°C until further bacterial DNA extraction.

Stable isotope analysis

Frozen microcosms were thawed at room temperature and copepods were collected and rinsed several times in MilliQ water to remove adhering particles. The copepods were transferred into a drop of MilliQ water in tin capsules (8 x 20 mm). The overall procedure was executed within 2 hours after thawing to minimize leakage of ^{13}C from the copepod body (Mourelatos et al. 1992, Moens et al. 1999d). Subsequently, the tin capsules were oven-dried overnight at 60 °C, pinched closed and stored under dry atmospheric conditions until analysis. Samples of the experimental food sources, i.e. fresh and freeze-dried diatom cells from the laboratory stock culture and from the ^{13}C-enriched cultures, were also analysed to check the initial labeling of the food sources.

Delta ^{13}C values ($\delta^{13}C$) were measured using an isotope ratio mass spectrometer (type Europa Integra) at the Davis Stable Isotope Facility (University of California, USA).

Uptake of ^{13}C is expressed as specific uptake ($\Delta\delta^{13}C = \delta^{13}C_{sample} - \delta^{13}C_{control}$). The control signal refers to the $\delta^{13}C$ value of *P. fulvofasciata* from the laboratory cultures, representing the isotope signal of the copepods prior to the start of the experiment. These data were further standardized according to Middelburg *et al.* (2000) by calculating excess (above background) ^{13}C and expressed as total uptake (I) in milligrams of ^{13}C per individual, calculated as the product of excess ^{13}C (E) and mean individual copepod biomass (organic carbon) per sample. Excess ^{13}C is the difference between the fraction ^{13}C of the control ($F_{control}$) and the sample (F_{sample}), where $F = {}^{13}C/({}^{13}C+{}^{12}C) = R/(R+1)$. The carbon isotope ratio (R) was derived from the measured $\delta^{13}C$ values as $R = (\delta^{13}C/1000 + 1) \times R_{VPDB,}$ with $R_{VPDB} = 0.0112372$ as $\delta^{13}C$ is expressed relative to Vienna Pee Dee Belemnite (VPDB).

As labeled diatoms had different initial $\delta^{13}C$ signatures (see 'laboratory cultures'), the total uptake per individual was further standardised taking into account the atomic % of ^{13}C in each food type. Differences

in diatom uptake by copepods between the various treatments were analysed with two-way analysis of variance (ANOVA) with the fixed factors 'food type' and 'food density', using the software package R, version 2.10.0 (R Development Core Team 2009) . *A posteriori* comparisons were carried out with the Tukey test using 95 % confidence limits. Prior to ANOVA, the Shapiro-Wilk test and the Levene's test were used to check for normality of the data and homogeneity of variances, respectively.

Bacterial community analysis by DGGE

Bacterial DNA was prepared through alkaline lysis (Baele et al. 2000) from subsamples of the original diatom stock culture (F cells), of the labeled culture (F* cells) and of both diatom cultures after lyophilization (D and D* cells). Bacterial DNA was also extracted from the fecal samples of each microcosm treatment (cell type x cell density). For the latter, the procedure from Baele *et al.* (2000) was slightly modified by reducing volumes of the reagents fourfold.

From each DNA extract an internal 194 bp 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′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3′) (Temmerman et al. 2003) coupled to the forward primer. PCR mixtures were prepared according to Temmerman *et al.* (2003). Respectively, 1 µl and 4 µl of DNA template from the diatom and fecal samples were included to achieve a successful PCR reaction. A touchdown PCR (Van Hoorde et al. 2008, 2009) 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. Fecal samples were subjected to a 'nested' PCR, i.e. the resulting PCR products were subjected to a second identical PCR. Subsequent denaturing gradient gel electrophoresis (DGGE) analysis using a 35-70 % gradient and staining of the gel were done as described by Van Hoorde *et al.* (2008, 2009).

Digitized DGGE gels were normalized and analysed by means of the BioNumerics programme (version 4.61, Applied Maths, Sint-Martens-Latem, Belgium). Calculation of the Jaccard correlation coefficient and application of Unweighted Pair Group Method with Arithmetic Mean (UPGMA) resulted in a dendrogram visualizing similarity between the band patterns of diatom and fecal pellet samples. Clusters were determined by visual inspection and by using the cluster cut-off method in the Bionumerics programme, a statistical tool to select most significant groups. Band profile similarities were also analysed by means of non-metric multidimensional scaling (nMDS).

RESULTS

Stable isotope signatures

Overall, specific diatom uptake by the copepods (expressed as $\Delta\delta^{13}C$; Fig. 1A) differed significantly among the various diatom diets and densities (2-way ANOVA, food: $p < 0.001$, density: $p < 0.01$, interaction: $p < 0.01$).

Between the single-food diets (Fig. 1A; Table 1; comparison of F*-D*), there was a highly significant difference in $\Delta\delta^{13}C$ values, except for F*l-D*h and F*l-D*m. Copepods clearly preferred the fresh cells as illustrated by the higher $\Delta\delta^{13}C$ values in comparison to the copepods of the corresponding freeze-dried treatments.

Table 1. Results of Tukey *post-hoc* tests for all treatment combinations. *: p<0.05, **: p<0.01, ***: p<0.001, -: not significant.

		single diet						mixed diet					
	food:conc	F*l	F*m	F*h	D*l	D*m	D*h	F*Dl	F*Dm	F*Dh	FD*l	FD*m	FD*h
single diet	F*l		-	*	***	-	*	***	***	*	***	***	***
	F*m			-	***	***	***	***	***	***	***	***	***
	F*h				***	***	***	***	***	***	***	***	***
	D*l					-	-	-	-	-	-	-	-
	D*m						-	-	*	-	-	***	-
	D*h							-	-	-	-	*	-
mixed diet	F*Dl								-	-	-	-	-
	F*Dm									-	-	-	-
	F*Dh										-	*	-
	FD*l											-	-
	FD*m												-
	FD*h												

Diatom cell density within a single-food diet did not strongly affect diatom assimilation by the copepod (Table 1; comparisons within F* and within D* treatments). For the fresh diet, a significant difference in diatom grazing was only recorded between the low and high cell density (F*l-F*h). In the case of freeze-dried cells, however, there was no effect of diatom density on the uptake by copepods.

Between the mixed-food treatments (Fig 1A; Table 1; comparison F*D-FD*), irrespective of the concentration, there were no differences in uptake of fresh and freeze-dried cells (except for F*Dh-FD*m). No selective grazing was observed, in contrast to the selectivity towards fresh diatoms in the single-food treatments. In comparison with the single-food diets (Table 1; comparison F*-F*D* and D*-FD*), grazing on fresh cells decreased when both cell types were offered simultaneously, whereas the uptake of freeze-dried cells in the single diet did not differ significantly from the uptake in the mixed diet (except for D*m-FD*m and D*h-FD*m). Within each mixed diet (Table 1; comparisons within F*D and within FD* treatments), diatom density did not interfere with diatom uptake, as was observed for the single foods.

After correction for the initial ^{13}C-enrichment levels of the food sources, the total carbon uptake per individual (Fig. 1B) was similar to the $\Delta\delta^{13}C$ pattern (Fig. 1A) except for the FD* treatment, due to the higher carbon content of the analyzed copepods in that treatment.

By summing the total carbon uptake per individual of the 2 mixed diet treatments, i.e. total carbon uptake of fresh cells (Fig. 1B; F*D) and total carbon uptake of freeze-dried cells (Fig. 1B; FD*), the overall uptake in the mixed diet was estimated (Fig. 1B; FD). Carbon uptake by copepods increased by one fifth when offered a mix of both cell types compared to assimilation of the fresh diet.

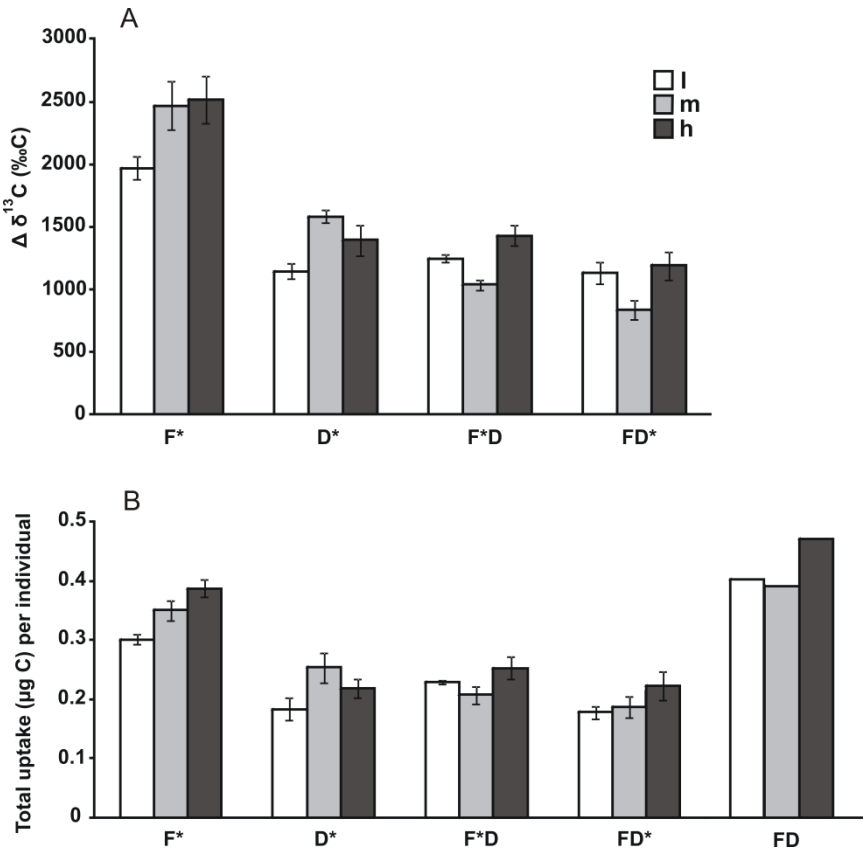

Fig. 1. Assimilation of ^{13}C (mean ± standard error) by *Paramphiascella fulvofasciata* after grazing on 4 enriched diatom diets (F* = fresh cells, D* = freeze-dried cells, F*D = mix of both where the fresh cells were enriched and FD* = mix of both where the freeze-dried cells were enriched) provided in 3 diatom cell densities (l = low, m = medium, h = high). Diatom uptake within each treatment is expressed as (A) $\Delta\delta^{13}C$ and (B) total uptake per individual relative to biomass and to stable isotope signature of the enriched food sources. FD shows the overall uptake of the mixed diet, after summing the assimilation from the treatments F*D en FD*.

Bacterial community on diatoms and fecal pellets

In the DGGE dendrogram representing the microorganism communities, three distinct clusters were defined (Fig. 2; cluster 1-3). Cluster 1 consists of fecal samples collected from the freeze-dried treatment (D*). Cluster 2 groups the bacterial communities on the fresh and freeze-dried diatoms. The bacterial communities on fecal pellets after feeding on fresh diatoms (F* or F*D) clustered together in cluster 3. Similarity between the clusters was ≤ 30 % and within the clusters ≥ 44 %. The same groups were retrieved in the nMDS plot (Fig. 3). The 'diatom cluster' (cluster 2) was more similar to the 'fresh fecal pellet cluster' (cluster 3) than to the 'freeze-dried fecal pellet cluster' (cluster 1).

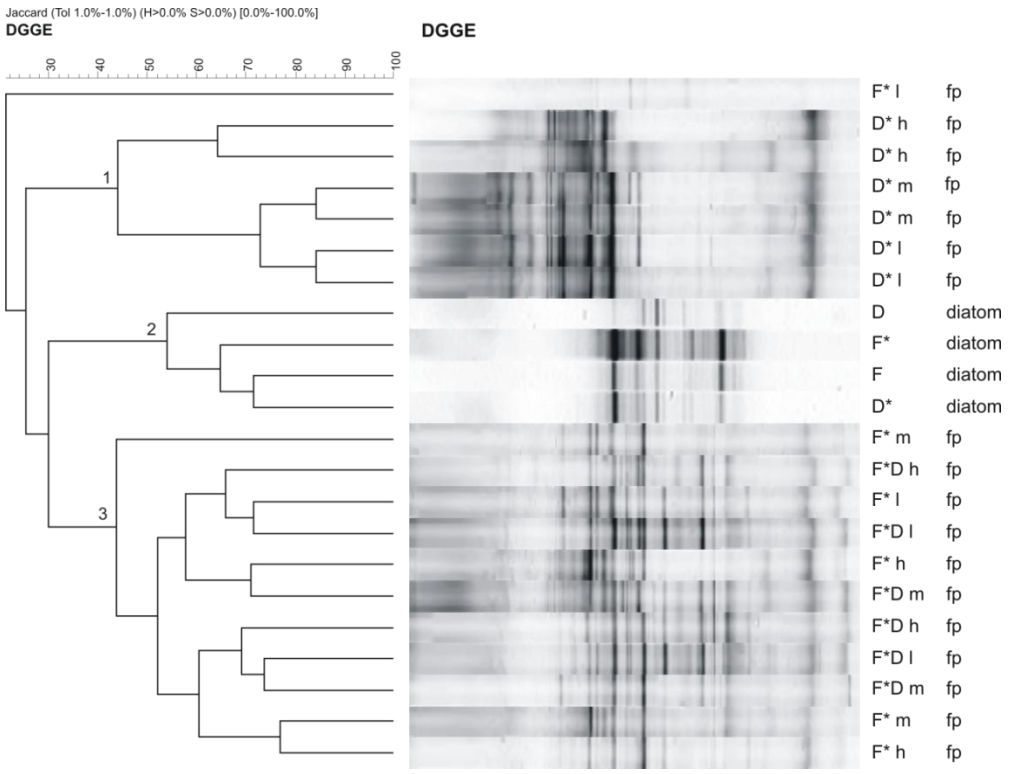

Fig. 2. Dendrogram (UPGMA) based on DGGE fingerprints of V_3 region amplicons (fragment of the 16S rRNA gene) representing bacterial communities associated with the diatom *Seminavis robusta* and with copepod fecal pellets (referred to as fp) collected from the experimental treatments containing diets F*, D* and F*D in densities l, m and h.

Bacterial assemblages on the diatoms clustered together (cluster 2), regardless of the freeze-drying and labeling of the diatom cells, with a similarity between 54 and 71 %. Differences in DGGE band profile intensities were notable. Labeled cells (F*, D*) showed more intense bands than the unlabeled cells (F, D). The samples of freeze-dried cells (D, D*) showed a weaker profile than its fresh equivalents (F, F*).

On the other hand, bacterial communities associated with fecal pellets were not at all alike, as illustrated by the presence of two fecal pellet clusters (cluster 1 and 3), with a similarity of 25 %. Within cluster 1, treatments were grouped according to the food densities as samples of the high density treatment differed from the two other densities. In cluster 3, however, no clear grouping of the different food densities and their replicates was observed.

One sample, F*l, was found isolated in the dendrogram due to the poor quality of the banding profile (top lane of Fig. 2). This sample will be ignored in further discussion, its replicate in cluster 3 is, however, still considered.

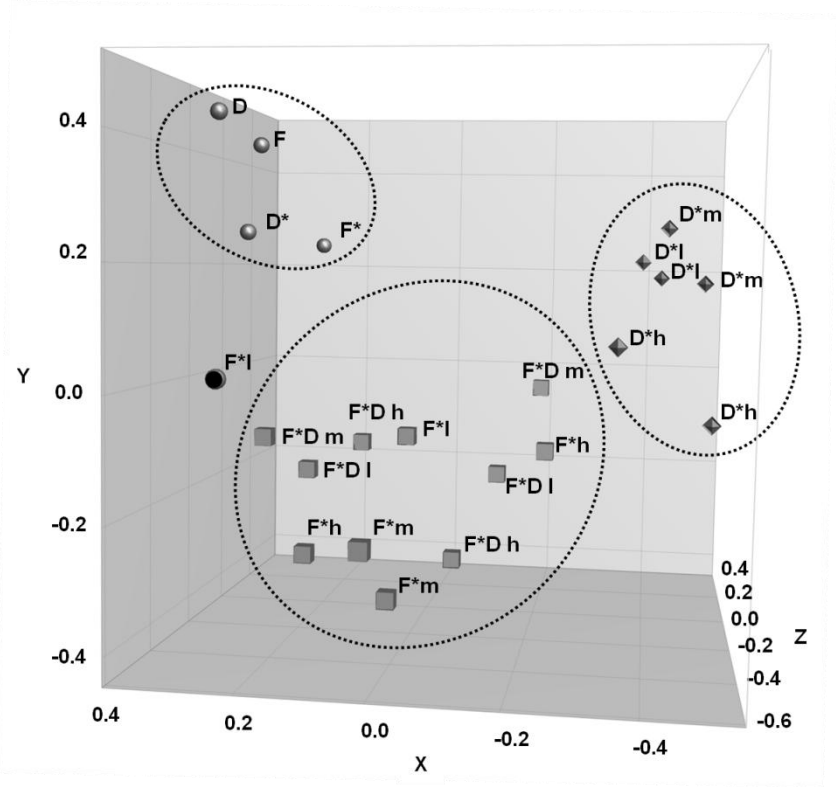

Fig 3. MDS ordination plot based on the relative band positions of the DGGE profiles.

DISCUSSION

Effect of freeze-drying on diatom palatability

P. fulvofasciata assimilated both fresh and freeze-dried *S. robusta* cells. The measured $\Delta\delta^{13}C$ values after 4 days of foraging on either of the diatom cell types were within the range of previous studies (De Troch et al. 2007, 2008) and together with the low copepod mortality rate confirms the suitability of this epipelic diatom species as a diet for *P. fulvofasciata*, even after preservation of the diatom.

A clear effect of the preservation status (fresh vs. freeze-dried) of the diatom cells on the feeding rate of *P. fulvofasciata* was, however, found. Fresh cells were significantly more assimilated by this harpacticoid copepod than freeze-dried cells. This selectivity toward a fresh diet was unlikely to be caused by morphological traits of the diatom, but presumably it was driven by chemical (and qualitative) traits of the cells and could be the result of active, i.e. ingestion of cells, as well as passive, through differential assimilation or digestibility, feeding preferences. To our knowledge, no morphological effect of freeze-drying on microalgae cells has been documented. Microscopic observations of the diatoms after freeze-drying and rehydration in seawater, showed that diatom frustules did not burst and frustules appeared intact. Frustules still contained the protoplast. The increase of the $\delta^{13}C$ value after freeze-drying, which is indicative of diffusion of the lighter ^{12}C from the diatom, may point toward the presence of small injuries of the diatom cell wall. Furthermore, lyophilisation is known as a better preservation technique than drying as it stabilizes biochemicals with limited damage thus maintaining food quality. Nevertheless, Albentosa *et al.* (1997) and Dobberfuhl and Elser (1999) reported a minor diminution of microalgae quality due to the freezing process, mainly attributed to modifications of protein content and elemental ratios of C, P and N. However, the elemental ratios were still in a range considered indicative of a favourable food quality.

The current study suggests that external characteristics of the diatom cell contributed to the difference in copepod assimilation of fresh and freeze-dried cells. The exterior of the diatom cells may have been modified by lyophilisation, for instance by the loss of exudates (e.g. extracellular polymeric substances) and the limited revival and/or loss of bacteria associated with the diatom frustules. A change in the external 'appearance' could have reduced ingestion (active selection) or inhibited digestion of the freeze-dried diatoms (passive selection) by the copepod. The high selective ability of copepods has been illustrated by their discrimination between live and dead cells of the same diatom species (DeMott 1988). That study further reported that calanoid copepods discriminate against axenic diatoms, in line with our hypothesis that the bacterial community associated with diatoms may be a more important determinant for diatom selection by copepods than the intrinsic nutritional value of the diatoms (Cowles et al. 1988, Cotonnec et al. 2001). Barofsky *et al*. (2010) and Cowles *et al.* (1988) found that copepods discriminate diatom cells depending on growth phase, which is associated with differences in both internal and external metabolite composition. Studies have repeatedly presumed food selection and ingestion rates of copepods to be driven by chemoreception or the presence of bacteria (Hicks 1977, Cowles et al. 1988, Moore et al. 1999, Jiang et al. 2002) and we believe that this is also the cause of the low assimilation of freeze-dried diatom carbon. Beside active selection by the harpacticoid, freeze-dried diatoms might have a lower digestibility. For clams it appeared that growth rates were coupled to the ingestion and absorption rates of the preserved diet rather than to the biochemical content of the food sources (Albentosa et al. 1997), suggesting that acceptability of the preserved diet is of greater importance in determining the nutritional quality of a preserved food.

In the mixed diet of preserved and living cells, no selective feeding was observed. *P. fulvofasciata* equally assimilated both cell types. Moreover, total assimilation of the mixed diet was much higher than assimilation of the preserved diet and even the fresh diet. This is consistent with our suggestion that the external traits of diatoms were important in determining active food selection by the copepods. Substitution of 50 % of the freeze-dried food by fresh diatoms, compensated for the lack of chemical stimuli of freeze-dried cells. A similar observation on reduced chemical-mediated selectivity (Cowles et al. 1988) of dead diatoms after mixing with living cells have been documented for the calanoid copepod *Acartia clausi* and ingestion rates increased substantially (Mayzaud et al. 1998).

Bacterial communities on diatoms and fecal pellets

The DGGE profiles of the bacterial communities differed between the fresh and the lyophilized diatoms, although both originated from the same culture. Bacterial richness (number of bands) on the freeze-dried cells was similar to that on the living cells. However, taking into account the semi-quantitative properties of DGGE, bacterial abundances, represented by band intensities, were different.

Part of the dissimilarity may have been caused by the additional washing step of the diatoms after lyophilisation to remove salt crystals. In addition, an artificial effect of the added carbon (^{13}C labelling) on the bacteria is observed (i.e. higher intensity and higher number of bands). The increased level of carbon sources in the diatom growth medium could have stimulated bacterial growth, directly or indirectly. However, DGGE band intensities should be interpreted with caution since the lack of standardization toward diatom biomass during bacterial DNA extraction could account for differences in band intensities. Moreover, viability of the diatom-associated bacteria after freeze-drying and rehydration is expected to be extremely low since no protective medium was used (Berner & Viernstein 2006).

Bacterial composition on fecal pellets depended upon diet and was very distinct from the bacterial communities on diatoms. Copepod fecal pellets are a growth substratum for a variety of heterotrophic bacteria (De Troch et al. 2010). Fecal pellet bacteria have been suggested to originate from the environment through colonization after egestion or to consist of transient or resident gut bacteria, i.e.

ingested bacteria that survived gut passage (Harris 1993, Hansen & Bech 1996). Based on our DGGE profiles, bacteria associated with diatoms contributed only a minor part of the bacterial richness found on fecal pellets, thus indicating that there was a substantial release of gut bacteria by the harpacticoid and not just an exchange of bacteria between copepod and the environment, irrespective of the experimental diet. The bacterial diversity on the fecal pellets and thus on the copepod gut lining, however, did differ according to the type of food ingested by the copepod. Hence, the ingestion of the diet, not its presence in the environment, was the main factor shaping the bacteriflora in the copepod digestive tract, as was documented already in terms of bacterial abundance (Tang 2005) and diversity (De Troch et al. 2010).

Fecal pellets produced after feeding on the lyophilised cells harbored a specific and relatively stable bacteriflora. The variability of the bacterial communities on fecal pellets after feeding on fresh diatoms was, however, pronounced. The introduction of metabolically active bacteria associated with the fresh diatoms into the gut likely gave rise to complex bacterial dynamics (e.g. competition for nutrients, space), diversifying the gut microflora. This assumption is in agreement with previous studies (Tang 2005, De Troch et al. 2010) and our fecal pellet analyses are complementary to the study of Tang *et al.* (2009) who deduced an effect of food on the gut biota indirectly through examination of the copepod body, using DGGE.

Bacterial enrichment due to grazing

At the beginning of the experiment, a similar bacterial community (i.e. the bacteria associated with the diatom cells) was introduced to all microcosms. Nevertheless, the bacterial richness on the fecal pellets at the end of the experiment deviated strongly from the original microcosm diversity, as shown by the bacterial diversity found on the fecal pellets. The egestion of fecal pellets introduced new bacteria into the experimental units (see also De Troch et al. 2010, who identified Alphaproteobacteria, Flavobacteria and Bacilli as bacteria solely found on fecal pellets). An increase in bacterial diversity was indirectly caused by the copepod grazing activity on diatoms (via fecal pellets) indicating that the presence of grazers induced shifts in environmental bacterial diversity.

Since harpacticoids seasonally switch between food sources (Lee et al. 1976, Hicks & Coull 1983), further research on copepod-bacteria interaction would be interesting. A top-down effect of copepod grazing on bacterial community composition in sediments is not inconceivable and can play a potential role in ecosystem functioning such as degradation processes and the biological pump.

Implications

The present study shows that the copepod *P. fulvofasciata* is able to discriminate between freeze-dried and fresh diatoms. This finding has important implications for laboratory experiments that use freeze-dried food. The use of preserved food for ecological experiments offers several advantages and in some cases may be required in case of limited access to living algae or for experiments in remote or extreme environments (e.g. deep sea) (Ingels et al. 2010). Based on our results, we can state that the grazing rates reported in studies using preserved food may deviate from the feeding rates on their natural food sources.

For aquaculture applications aimed at upscaling of harpacticoid cultures, our results suggest that a preserved diatom food may result in lower copepod growth rates. Long-term experiments are necessary to analyse the effects of preserved diets on copepod fitness, especially since adverse effects of a diet may not appear within the first week (Irigoien et al. 2002). The observation of a lower assimilation of the preserved food together with previously published indications that freeze-dried diatoms may have a lower nutritional value (Albentosa et al. 1997) urges some caution as to the use of preserved diatoms as food in aquaculture. However, this paper is the first to show an elevated assimilation of a mixed diet

consisting of preserved and fresh diatoms (at least as high as the uptake of a completely fresh diet). Changes in nutritional quality of freeze-dried diatoms should be profoundly studied on a biochemical level as this would provide baseline information for 'engineering' balanced preserved diets consisting of multiple freeze-dried food sources. In addition, information on the diversity, composition and abundance of the microbial assemblages associated with the copepod food should be included since bacteria may impact harpacticoid feeding behaviour. Our study underlines the potential of freeze-dried, or in general dead, diatom cells as food for copepods. This corresponds to what can be expected what happens during spring blooms when diatom cells of different status reach the benthos and where the cell condition may be important for the grazing selectivity of e.g. copepods. This all suggests that copepods, as well-known diatom grazers, have the potential to cope with live and dead diatom cells both under natural and experimental conditions.

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