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Dynamics of pigment degradation by the copepodite stage of *Pseudodiaptomus euryhalinus* feeding on *Tetraselmis suecica*

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Abstract Short-term (3 h) changes in concentration of chlorophylls and their derivatives in stage V *Pseudodiaptomus euryhalinus* and their fecal material were followed by HPLC during a 24 h experiment. Copepodites were fed with the prasinophyte *Tetraselmis suecica*. Intact chlorophyll *a* and *b* were found in animals and fecal material and had similar dynamics of accumulation over time. The extent of transformation of chlorophyll *a* and *b* to colorless compounds was different with chlorophyll *a* being more extensively degraded. Additionally, several chlorophyll derivatives (pheophytin and pyropheophytin-like pigments) were found. Pyropheophytin *a* was the most abundant followed by pheophytin *b*, pheophytin *a*, and pheophorbide *a*. Relative amounts of pheopigments were different in copepodites and fecal material, and pheophytin *a*, pheophorbide *a*, and pheophytin *b* concentrations were low and variable. The amount of ingested chlorophyll recovered as chlorophyll *a* and its derivatives in fecal and copepodite pools was generally low ($< 5\%$), with one exception occurring after 9 h, when it accounted for $> 70\%$. These data suggest individual pheopigments are produced at different rates and that chemical or enzymatic mechanisms in the gut of copepodites act on the two chlorophylls in different ways.

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Introduction

The use of pigments and their derivatives as tools to study heterotrophic activity has became an active field of research in the last two decades. This can be associated with the introduction of rapid (< 30 min) analytical techniques for characterizing algal chlorophyll and carotenoid pigments and their breakdown products (Mantoura and Llewellyn 1983) and, more recently, with the discovery of pigment derivatives associated with heterotrophic activity (Repeta and Gagosian 1987; King 1993; Strom 1993). Furthermore, progress has been made in determining the relationship between the amount of pheopigment produced and the amount of phytoplankton ingested (Head and Harris 1996; Stevens and Head 1998). The experimental results in the cited papers have shown that phytoplankton pigments and their derivatives can be used to elucidate pathways of carbon flux in aquatic systems (Repeta and Gagosian 1984, 1987). Most studies have emphasized the production of the bulk of pheopigments (chlorophyll derivatives) and the destruction of chlorophyll *a* (Conover et al. 1986; Peterson et al. 1990; Penry and Frost 1991; Head and Harris 1996; Stevens and Head 1998) and short-term changes of individual derivatives of chlorophylls have generally been overlooked.

In our work, we tracked pigment changes in the copepodite *Pseudodiaptomus euryhalinus* and its fecal material during feeding with *Tetraselmis suecica* to study degradation of pigments over short (3 h) sequential time periods. Specifically, we wanted to understand the dynamics of degradation of individual chlorophylls and the production of their derivatives.

Materials and methods

The copepod *Pseudodiaptomus euryhalinus* was isolated from tide pools in the Ensenada de La Paz, Gulf of California (Payan-Aguirre 1994) and cultured in 200-l tanks with 20,000 cells ml^{-1} of *Tetraselmis suecica* as food. *T. suecica* was grown in *f*/2 medium

(Guillard 1975) under a 12 h light:12 h dark period at 25°C. After egg release, eggs were separated and reared under the same conditions as adults. Copepodite stage V was reached 7 days after egg hatching. Copepodites in stage V were used in this study.

Experimental procedures

For experiments 21 plastic bottles (3 l capacity) containing 500 copepodites l^{-1} and 20,000 cells ml^{-1} of *T. suecica* (experimental bottles) and 6 bottles containing only 20,000 cells ml^{-1} of *T. suecica* (control) were maintained in dim light at 25°C. Before the experiment, copepodites were incubated 1 h in filtered seawater (FSW) to allow them to empty their guts. Every 3 h, three experimental bottles were removed. From each of these, 300 ml of seawater was screened off using a 200 µm mesh to collect the copepodites and a 20 µm mesh sieve to collect the fecal pellets. These were washed from the sieves with FSW and concentrated on 25 mm GF/F filters. Screened seawater from the experimental (100 ml) and control bottles (10 ml) was also recovered on GF/F filters for pigment analysis. Only samples from time 0, 12, and 21 h were analyzed from the control bottles. All filters were immediately frozen (–20°C). Additionally, formaldehyde was added to 100 ml of screened seawater from the experimental and control bottles (final concentration 4%) for cell counting.

Pigment analysis

Pigments from the filter were extracted in 2 ml of 100% acetone, ground in glass tubes, and then centrifuged. The extract (0.2 ml) was mixed with 0.1 ml of 0.5 N ammonium acetate and injected through a 100 µl loop onto a reverse-phase C8 HPLC system (Hewlett Packard series 1100), equipped with a diode array detector. HPLC conditions were as described in Vidussi et al. (1996). To summarize, a hypersil MOS 10-cm, 4.6-mm (i.d.), C8 column was used with the following solvent A (MeOH) and solvent B (MeOH: 0.5 N aqueous ammonium acetate, 70:30% v/v) gradient (min, percentage of solvent A, percentage of solvent B): 0, 75, 25; 1, 50, 50; 15, 0, 100; 18.5, 0, 100; and 19, 75, 25. Pigment identification was made by comparing retention times and spectral characteristics with those of commercial pigment standards (International Agency for ^{14}C determinations, Denmark) and derivatives of chlorophyll *a* and *b* (pheophorbide and pheophytin) produced by us according to Jeffrey (1997). Once the pheopigments were produced, they were quantified by using the specific extinction coefficient given by King (1993) and used to obtain the response factors (HPLC peak area/pigment mass) according to Mantoura and Repeta (1997). Pyropheophytin *a* was quantified by using the response factor of pheophytin *a*, because their spectra closely match.

Results

Concentration of *Tetraselmis suecica* cells showed no significant changes in the control bottles (Fig. 1a). The same was true for the chlorophyll concentrations (Fig. 1b). In the experimental bottles, cell numbers decreased rapidly to almost one-third of their original values after 6 h (Fig. 1c) and, thereafter, remained relatively constant. Chlorophyll concentrations showed dynamics similar to those of cell numbers (Fig. 1d). The changes in chlorophyll *a* (chl *a*) and chlorophyll *b* (chl *b*) in the experimental bottles were significantly related to changes in cell number (Fig. 2).

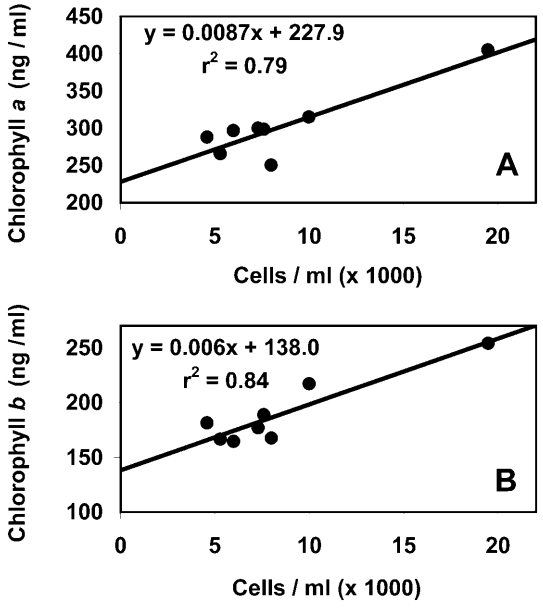


Fig. 2a, b *Tetraselmis suecica*. Relationship between cell number and chlorophyll *a* (**a**) and chlorophyll *b* (**b**). Line represents the best-fit equation (r^2 correlation coefficient). Both relationships are significant at $\alpha = 0.05$

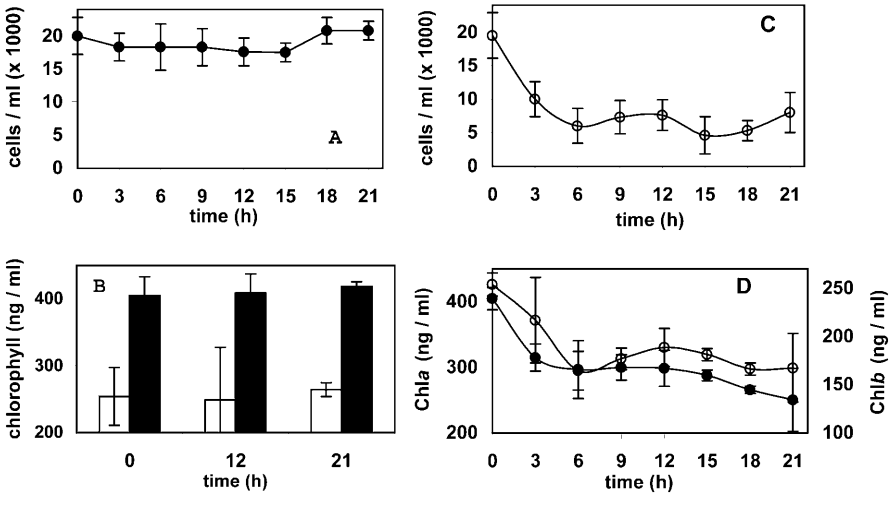


Fig. 1a–d *Tetraselmis suecica*. Variation of cell numbers and chlorophylls in control (**a, b**) (*filled bars* chlorophyll *a*; *open bars* chlorophyll *b*) and in experimental bottles (**c, d**)(*filled circles* chlorophyll *a*; *open circles* chlorophyll *b*) during experiment (*vertical bars* standard deviation)

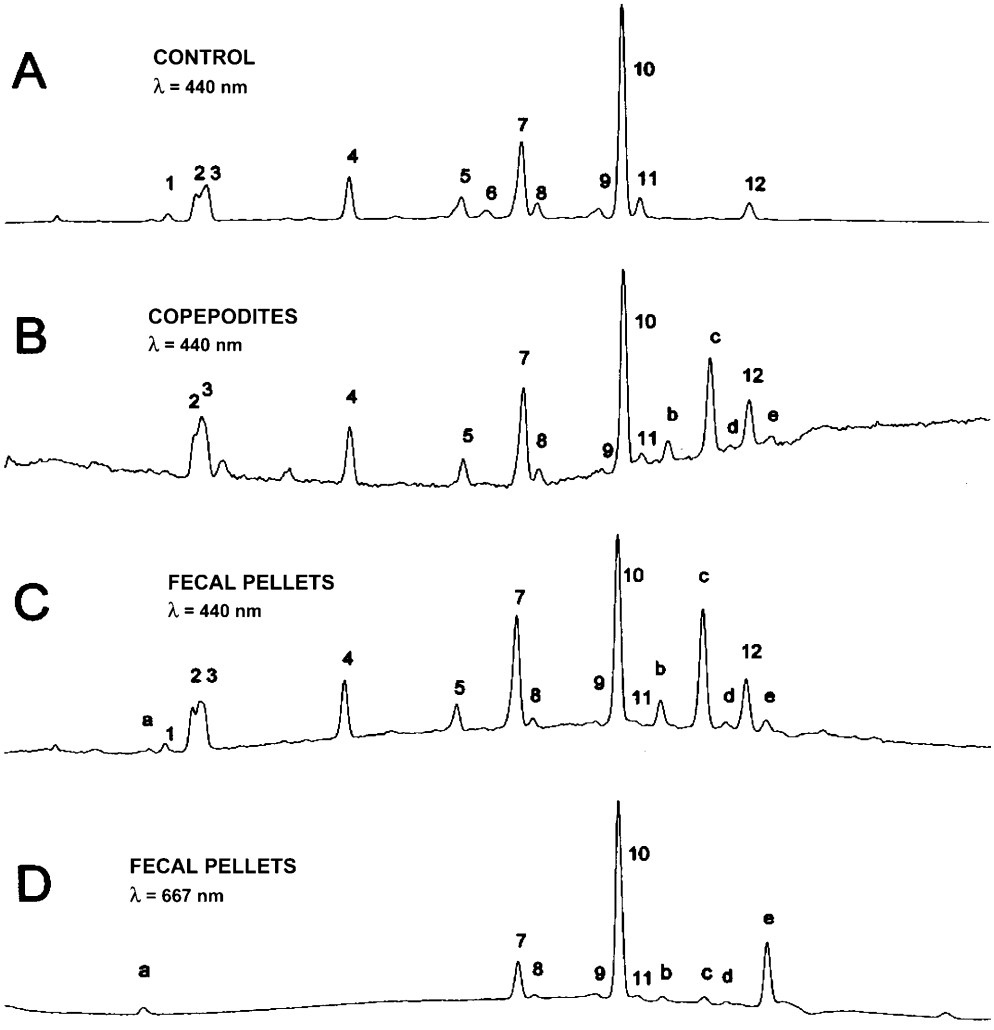


Fig. 3a–d *Tetraselmis suecica*, *Pseudodiaptomus euryhalinus*. Absorbance chromatograms of representative control *T. suecica* (**a**), copepodites (**b**), and fecal material (**c, d**) samples. Peak signals were recorded at 440 nm (**a, b, c**) and at 667 nm (**d**) [Pigments: *1* trans-neoxanthin-like; *2* trans-neoxanthin; *3* violaxanthin; *4* lutein; *5* unknown pigment; *6* chlorophyll *b* (allomer); *7* chlorophyll *b*; *8* chlorophyll *b* (epimer); *9* chlorophyll *a* (allomer); *10* chlorophyll *a*; *11* chlorophyll *a* (epimer); *12* α-carotene. Chlorophyll derivatives: *a* chlorophyllide *a*?; *b* unknown pheophythin-like; *c* pheophytin *b*; *d* pheophytin *a*; *e* pyropheophytin *a*]. Chromatograms are not in the same absorbance scale. Only chlorophylls and their derivatives are considered in this paper

Both chlorophylls appeared in copepodites and their fecal material (Fig. 3) as did chlorophyll derivatives eluting before (pheophorbide-like) and after (pheophytin-like) chl *a*. The on-line spectra of the derivatives showed two types: (1) with maximum absorbances at 410 and 666 nm (peaks b, d, e), and a Soret (blue)/red ratio of 1.65; and (2) with maximum absorbances at 436 and 654 nm (peak c), and a Soret/red ratio of 4.89 (peak c). These spectral characteristics are nearly identical with those of known degradation products of chl *a* and chl *b* (Jeffrey et al. 1997), specifically pheophytin *a* (peak d), pyropheophytin *a* (peak e), and pheophytin *b* (peak c). We were unable to identify peak b, but its occurrence was irregular, and it will be neglected hereafter.

Pigments in copepodites and fecal material

To facilitate comparison between copepodites and fecal pellets, pigment content data are expressed as the amount of pigments per copepodite and, for the fecal pellets, contained in2 ml of seawater occupied by one copepodite (i.e. pigments excreted by a single copepodite). Chlorophyll concentrations reached their maximum values in copepodites at 3 h (2.1 ng chl *a* and 1.8 ng chl *b* copepodite^{-1}) and remained relatively constant for the following 6 h (Fig. 4a). Then, they decreased steadily with time to reach values <1.5 ng copepodite^{-1}. Chlorophylls accumulated in fecal material to reach a small plateau between time 6 and 9 h and a maximum at between 12 and 18 h after feeding had started (Fig. 4b).

Among the chlorophyll degradation products, pyropheophytin *a* (pphtin *a*) was the most abundant in both copepodites and fecal material (Fig. 5), followed by pheophytin *b* (phtin *b*), pheophytin *a* (phtin *a*), and pheophorbide *a* (pbid *a*). In copepodites, pphtin *a* increased almost linearly to reach a maximum value of 1.8 ng copepodite^{-1} at 6 h and, thereafter, decreased to a value of 0.7 ng copepodite^{-1} (Fig. 5c). Phtin *a*, phtin *b*, and pbid *a* concentrations were low (never reaching values >0.2 ng copepodite^{-1}) and variable (Fig. 5a, c, e). All pheopigments accumulated in fecal pellets, as did chlorophylls, and in general reached a maximum at 12 h (Fig. 5b, d, f). Thereafter, only pphtin *a* and phtin *b* showed a decrease towards the end of the incubation period (Fig. 5d, f).

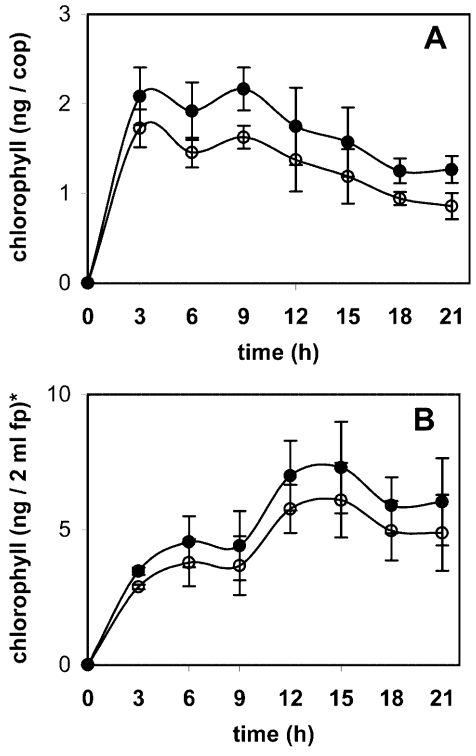


Fig. 4a, b *Pseudodiaptomus euryhalinus*. Chlorophyll variation in copepodites (**a**) and fecal material (**b**) during experiment (*filled circles* chlorophyll *a*; *open circles* chlorophyll *b*; *vertical bars* standard deviation). Pigment concentrations in fecal material are those contained in 2 ml of seawater occupied by one copepodite

Percentages of the ingested chlorophyll recovered as chlorophyll and its derivatives (expressed as percent of equivalent weight of chlorophyll) in fecal material and copepodites were in general $< 5\%$ (Table 1), with the exception of one value recorded between 9 and 12 h, when chl *a* appeared in fecal material representing $> 70\%$ of that ingested. Chl *b* and phtin *b* concentration recoveries were $< 5\%$ of the ingested chl *b* (Table 1). The overall degree of chlorophyll destruction (expressed as percent of equivalent weight of chlorophyll) and ingestion rate data showed a very high efficiency of pigment destruction, almost 100% at ingestion rates > 3.5 ng chl *a* copepodite^{-1} h^{-1} (Fig. 6).

Discussion

Our experimental results show that a fraction of the chlorophylls ingested by *Pseudodiaptomus euryhalinus* copepodites passes through their guts and appears intact in fecal pellets (Fig. 1). These results are consistent with observations of others (Nelson 1989, 1993; Roy and Poulet 1990; Head and Harris 1992; Kleppel 1998; McLeroy-Etheridge and McManus 1999). Of the chl *a* derivatives, pheophytin, pyropheophytin, and pheophorbide were produced, while, of the chl *b* derivatives, only pheophytin was found. These pheopigments have also been reported previously (Head and Harris 1992, 1996; Table 6 in Strom 1993; McLeroy-Etheridge and McManus 1999), although the dynamics of their appearance were very particular. In copepodites, phtin *a* was present in low and more-or-less constant concentrations during the experiment. Pphtin *a* was more abundant, with an almost linear increase between 0 and 6 h and a decrease thereafter. Finally, pbid *a* was produced in low, but variable concentrations. Pheopigments accumulated in fecal material to reach a plateau concentration at 12 h. A decrease was observed for pphtin *a* and phtin *b* at the end of the experiment.

Although transformation of chlorophylls by phytoplankton enzymes was documented almost two decades ago (Owens and Falkowski 1982), its role as a factor in chlorophyll destruction during copepod grazing was only recently considered (Head and Harris 1996; Stevens and Head 1998). According to these authors, enzymes derived from both the copepod and ingested phytoplankton can destroy or transform ingested chlorophylls. Presumably, enzymatic reactions occur prior to the entry of chlorophylls into the copepod gut, as the cells are macerated by the copepods. This enzymatic transformation of chlorophylls is related to the ingestion rate (Head and Harris 1996; Stevens and Head 1998) and can result in variable efficiency of transformation depending on the concentration of enzymes and substrate (phytoplankton chlorophyll) presence in the foregut. In long-term experiments (i.e. 12–24 h) the fraction of ingested chl *a* "destroyed" by copepods during feeding is variable (from almost 0% to 100%; Lopez et al. 1988; Penry and Frost 1991; Head and Harris 1992, 1996; Stevens and Head 1998; McLeroy-Etheridge and McManus 1999). This destruction is also variable over a short-term period (hours), as shown by our data (Table 1) and in agreement with the experimental data of Head (1992). Interestingly, chl *a* and chl *b* derivatives followed a similar trend: accumulation in fecal material, followed by decreases towards the end of the experiment (Fig. 4). These dynamics are consistent with observations of the decrease in phytoplankton levels (Fig. 1) and suggest that active grazing activity on *T. suecica* occurred mostly during the initial hours of the experiment. Subsequently, there may have been ingestion of the fecal material and further breakdown of pigments to colorless compounds. Copepod ingestion of its own fecal material is possible (Lopez et al. 1988; Head and Harris 1996).

An additional degradation of ingested chlorophylls may occur in the midgut region; B-cells are located in this region of the gut (Arnaud et al. 1980), which absorb luminal substances (Brunet et al. 1994). Once inside B-cells, food has an enzymatic as well as an acidic contact, because of the presence of acid enzymes in the vacuolar apparatus (Brunet et al. 1994). Here, chlorophylls could suffer a transformation to pheophytin, because they are labile under acidic conditions (Daley and Brown 1973; Jeffrey 1997). B-cells accumulate partially digested material and eventually expel the cell contents into the gut

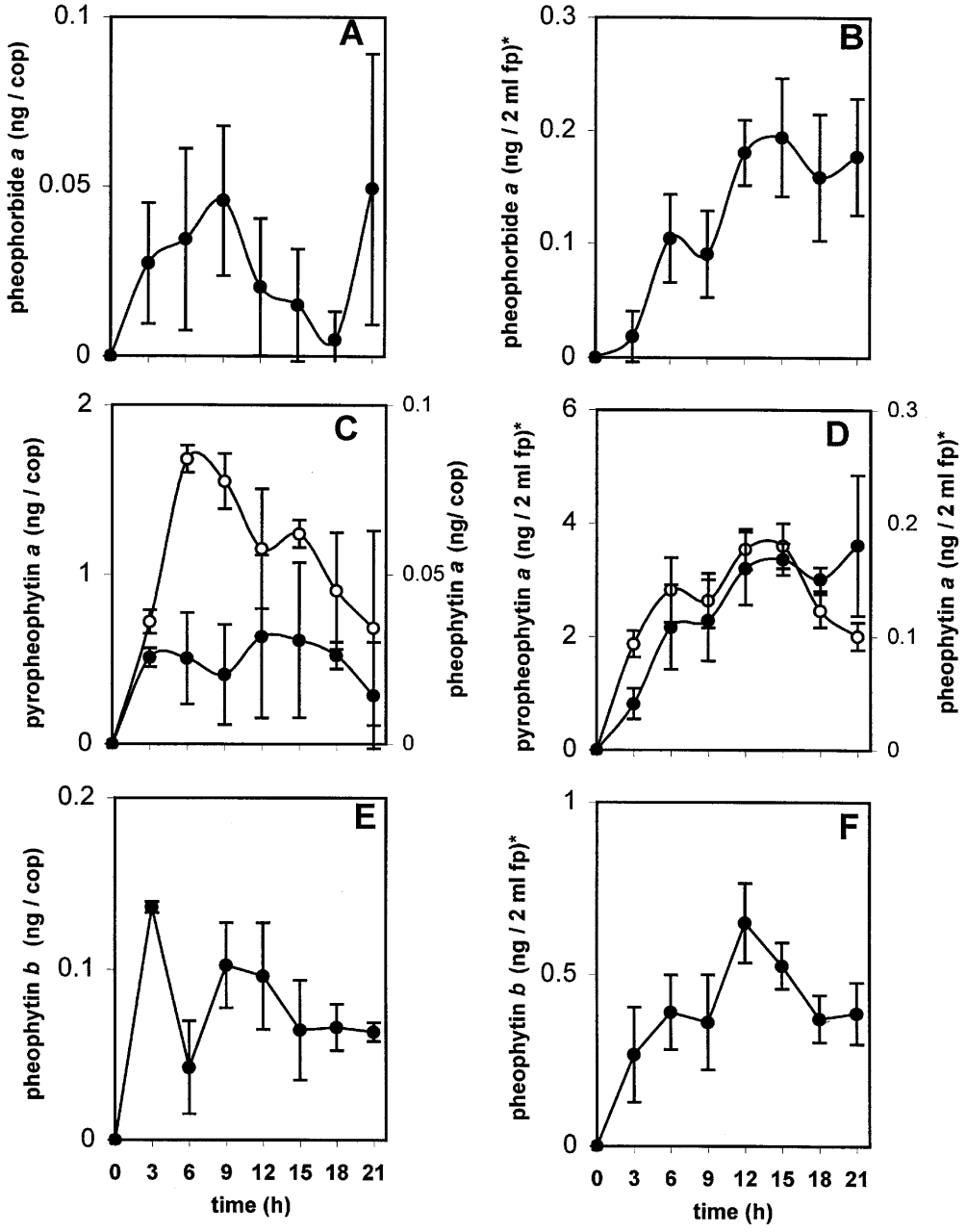


Fig. 5a–d *Pseudodiaptomus euryhalinus*. Variation of chlorophyll derivatives in copepodites (**a, c, e**) and fecal material (**b, d, f**) during experiment. (*filled circles* pheophytin *a*; *open circles* pyropheophytin *a*; *vertical bars* standard deviation). Pigment concentrations in fecal material are those contained in 2 ml of seawater occupied by one copepodite

to be reabsorbed by R-cells. For *Calanus helgolandicus*, the time elapsed was about 2–3 h for extensive liberation of material and 7 h for complete depletion of B-cell content after the onset of feeding (Nott et al. 1985). After this time, food digestion may depend not on ingestion, but on the rate of B-cell replacement (Hasset and Landry 1988). If these digestive processes hold for the copepodite stage of *P. euryhalinus*, they would explain the bulk of pheopigment dynamics observed in the fecal material. The periods of high-efficiency transformation of chlorophyll to colorless products (before and after the 9–12 h period) probably are linked to the activity of both copepods and algal enzymes, while the low efficiency of chl *a* degradation is perhaps due to the depletion of B-cell contents. Nott et al. (1985) noted that the pellet content of the copepod *C. helgolandicus* contains mostly undigested phytoplankton cells 7 h after feeding commenced. Chl *a* represented >60% of the ingested pigment in our experiment during the 9–12 h period.

To our knowledge, short-term dynamics of individual pheopigment types during copepod grazing has not been previously reported, and most of the literature is related to the total bulk of pheopigments (Head 1992; Mayzaud and Razouls 1992; Tirelli and Mayzaud 1998). Of these

Table 1 *Pseudodiaptomus euryhalinus*. Percentage of the ingested chlorophyll *a* recorded as chlorophyll and derivatives in fecal material and copepodites. Values are percentage of the ingested chlorophyll in chlorophyll equivalent (*chl a* chlorophyll *a*; *pbid a* pheophorbide *a*; *phtin a* pheophytin *a*; *pphtin a* pyropheophytin *a*; *chl b* chlorophyll *b*; *phtin b* pheophytin *b*)

Period	Pigments (as %)					
	Chl *a*	Pbid *a*	Phtin *a*	Pphtin *a*	Chl *b*	Phtin *b*
Fecal material						
0–3 h	1.6	0.0	0.0	0.6	4.7	0.4
3–6 h	2.4	0.4	0.0	0.0	1.0	0.1
6–9 h	0.0	0.0	0.0	0.0	0.0	0.0
9–12 h	72.7	4.7	0.3	2.3	0.0	0.0
12–15 h	1.5	0.1	0.0	0.0	2.0	0.0
15–18 h	0.0	0.0	0.1	0.0	0.0	0.0
18–21 h	0.0	0.1	0.0	0.0	0.0	0.0
Copepodites						
0–3 h	1.0	0.0	0.0	0.4	2.8	0.2
3–6 h	0.0	0.0	0.0	2.9	0.0	0.0
6–9 h	0.0	0.0	0.0	0.0	0.0	0.0
9–12 h	0.0	0.0	0.4	0.0	0.0	0.0
12–15 h	0.0	0.0	0.0	0.5	0.0	0.0
15–18 h	0.0	0.0	0.0	0.0	0.0	0.0
18–21 h	0.0	0.2	0.0	0.0	0.0	0.0

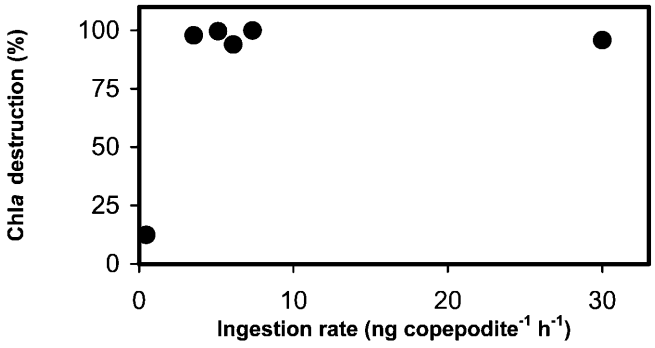
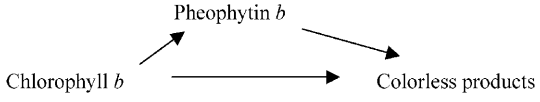


Fig. 6 *Pseudodiaptomus euryhalinus*. Relationship between ingestion rate (ng copepodite^{-1} h^{-1}) for each time period when the copepodites were actually feeding and the overall degree of pigment destruction for the same period

works, Tirelli and Mayzaud (1998) reported a good fit of the chlorophyll destruction and ingestion rate data to a Michaelis–Menten equation. Fitted parameters showed that high levels of pigment destruction (>90% of ingested pigment) were rapidly reached. A similar trend was observed in our data, with almost 100% of chl *a* destroyed at ingestion rates >3.5 ng copepodite^{-1} h^{-1} (Fig. 6). However, the above-mentioned results contrast with those obtained when longer incubation period intervals are used (Head and Harris 1996; Stevens and Head 1998). We have no explanation for these contrasting results, but differences in temperature, phytoplankton, and zooplankton species could be involved.

In the case of individual pheopigments, our results show that the pheopigment types had different dynamics in the gut of the copepodite, suggesting that lability of the chlorophyll molecules or chemical conditions within the gut of the copepodite may regulate its concentration. The rapid transformation of the more labile molecule of phtin *a* to the more stable molecule of pphtin *a* may maintain its low but quasi-constant concentration during the experimental time (Fig. 5), or diverse mechanisms of transformation (enzymes, acidic conditions) could be acting at different rates. Of course a combination of both is also possible.

In contrast with the derivatives of chl *a*, only one byproduct of chl *b* was recorded, phtin *b* (Fig. 3). This implies that the mechanisms involved in the destruction and transformation of chl *b* are different from the chl *a*. This is quite surprising because the difference between chl *b* and chl *a* is that in chl *b* a formyl group replaces the methyl group at C-3 on the macrocyclic ring (Rowan 1989). This molecular difference results in the higher lability of chl *a*, as suggested by the lower chl *a*/chl *b* ratio recorded in the gut and fecal pellets (average 1.6) when compared with the chl *a*/chl *b* value of the algae (2.1), and in a simpler degradative pathway (



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The efficiency of transformation to colorless or degradation products may depend on the type and quantity of food, as shown recently by McLeroy-Etheridge and McManus (1999) for adult females of *Acartia tonsa* fed on algal diets of *Tetraselmis* spp. and *Dunaliella terciolata*.

In summary, our data show that individual pheopigments are not produced at the same rate and underscore the complexity of chlorophyll biodegradation by copepods. Knowledge of the mechanisms involved in this transformation is needed if pigment derivatives are to be used as tools to calculate phytoplankton grazing by heterotrophic organisms.

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