Discrimination against 4-methyl sterol uptake during steryl chlorin ester production by copepods

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Abstract

To investigate possible reasons for the discrepancy between the abundance of 4-methyl components in free sterols and steryl chlorin esters (SCEs) which is commonly observed in sediments, experiments in which the copepod *Calanus helgolandicus* grazed on the dinoflagellates *Prorocentrum micans* and *Alexandrium tamarense* were carried out. With *P. micans* all the algal sterols were esterified as SCEs in the faecal pellets, but there was clear discrimination against the uptake of 4-Me sterols. This discrimination was also evident with *A. tamarense*. To investigate changes in the faecal pellet SCE and the free sterol distributions during pellet ageing in the *P. micans* experiment, portions were allowed to stand for up to 29 days in seawater in the dark. Although degradation of SCEs occurred, their esterified sterol distribution remained unchanged. The free sterols were degraded more rapidly than the SCEs and changes in the distribution were observed. This provides further laboratory evidence that sedimentary SCE sterols are more robust markers of phytoplankton communities than the corresponding free sterol distributions. Comparison of the SCE abundance in sterilised and unsterilised pellets indicated that the degradation was a result of microbial activity. © 2000 Elsevier Science Ltd.. All rights reserved.

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1. Introduction

Steryl chlorin esters (SCEs) are abundant chlorophyll (chl) biotransformation products with an almost ubiquitous presence in marine and lacustrine sedimentary environments (Eckardt et al., 1991, 1992; King and Repeta, 1991; Pearce et al., 1998). Field studies have indicated that they are formed in the water column as a result of zooplankton grazing (King and Repeta, 1991, 1994; King and Wakeham, 1996) and laboratory studies

have shown them to be produced when a copepod grazed on a diatom and on a prasinophyte (Harradine et al., 1996a; Talbot et al., 1999a,b). Their abundance in sediments relative to free sterols varies and a range of values from 0.02 to 6.2 has been reported (Pearce et al., 1998).

Comparison of the free and SCE sterol distributions in a number of sediments has highlighted consistent differences in the abundance of 4-methyl vs. 4-desmethyl components (King and Repeta, 1991, 1994; Eckardt et al., 1992; Pearce et al., 1998). For example, King and Repeta (1994) found that only 20% of the free sterols in a surficial sediment from the Black Sea were 4-desmethyl components, whereas 99% of the SCE sterols were 4-desmethyl components. The SCE sterol distribution also corresponded favourably with the distribution in the total flux of free sterols to the sediment, which consisted of 98% 4-desmethyl sterols. This difference

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was ascribed to the greater resistance of the free 4-methyl components to biodegradation (see also Pearce et al., 1998).

Sterols containing a 4-methyl substituent (e.g. dinosterol) are of particular importance as they are largely derived from a single source, the Dinophyta (dinoflagellates), although they are not found in all species. They are commonly used as indicators of dinoflagellate input to sediments; also, the occurrence of the resulting alkane, dinosterane (**I**, see Appendix), is taken as evidence for a contribution of organic matter from dinoflagellates to ancient sediments and oils and its presence in high abundance is generally considered to indicate a marine origin for the organic matter (Summons et al., 1992, and references therein). It should be noted, however, that there have been a number of reports of 4-methyl sterols in haptophytes from the class Pavloales (e.g. Conte et al., 1994, and references therein). A number of 4-methyl sterols, including dinosterol or its epimer, have also been identified in a marine diatom (e.g. Volkman et al., 1993).

As part of a wider study of SCE formation during herbivory we have carried out feeding experiments using two dinoflagellates in order to further investigate possible reasons for the discrepancy between the distributions in sedimentary SCE sterols and free sedimentary sterols. The main, large scale experiment, involving the copepod *Calanus helgolandicus* feeding on *Prorocentrum micans*, was designed to investigate: (i) the production of SCEs by copepods grazing on a dinoflagellate, (ii) any changes in distribution and abundance of SCE sterols in faecal pellets as they undergo ageing, (iii) changes in the faecal pellet free sterol distribution during ageing and (iv) the type of degradation (i.e. microbial vs. chemical). The second, small scale experiment involved the same copepod and *Alexandrium* (formerly *Gonyaulax*) *tamarense*.

2. Experimental

2.1. Feeding experiments

The *P. micans* experiment was carried out as for a large scale diatom experiment described previously (i.e. 16×5 l feeding beakers and two algal control beakers; Talbot et al., 1999a) except for the following differences. The cell density in the feeding beaker and algal control was 700 cells ml⁻¹ and a total of 100 stage V and females of *C. helgolandicus* were added to each beaker. After 48 h, the animals were removed by filtering through a 500 µm mesh and the faecal pellets were separated from any remaining algal cells by filtration through a 35 µm mesh. Any copepod eggs retained with the pellets were removed during microscopic examination. Pellets from all 16 feeding beakers were combined

and made up to a volume of 3 l in filtered seawater (0.6 µm). Three aliquots (50 ml) were taken for microscopic counting to determine the average number of pellets in each aliquot. A further 14 aliquots (200 ml each) of suspended pellets were taken for ageing experiments. Four were filtered immediately, four were aged in filtered seawater in the dark at constant temperature for 8 days, four for 29 days, and two were sterilised with HgCl₂ and aged for 29 days. Before solvent extraction, a synthetic C₃₂ hopanol chlorin ester ([22*R*]-30α,30β-dihomohopan-30β-yl pyropheophorbide *a* ester; Haradine et al. 1996b) and pregn-5-en-3β-ol were added to the pellet samples as internal standards. The algal control was extracted at the same time as the fresh pellets (i.e. after 48 h).

The experiment with *A. tamarense* was performed in the same way but on a smaller scale (six feeding beakers, one algal control) at a feeding cell density of 640 cells ml⁻¹ with 90 stage V and females of *C. helgolandicus* added to each feeding beaker. After 48 h the pellets from four of the beakers were collected immediately by filtering onto a GF/F glass fibre filter. Pellets from the remaining two beakers were aged in filtered seawater for 30 days and collected as above.

2.2. Extraction and fractionation

Details of the extraction and fractionation procedures are reported elsewhere (Talbot et al., 1999a). In brief, samples were extracted exhaustively with acetone until the extracts were colourless. Total extracts were examined for chlorins and sterols were isolated by thin layer chromatography using sitosterol as a standard and were analysed as the TMSi ethers.

2.3. High performance liquid chromatography–mass spectrometry (HPLC–MS)

HPLC–MS was performed as described previously (Talbot et al., 1999a) except for the use of an on-line Waters 996 PDA detector using Millenium³² software to provide electronic spectra of individual components from 350 to 700 nm during analysis of the *P. micans* samples. Pigments were assigned as described previously (Talbot et al., 1999a) and quantified from HPLC peak areas (400 nm).

2.4. Gas chromatography and gas chromatography–mass spectrometry (GC and GC–MS)

GC analyses and sterol quantitation were performed using a Carlo Erba 5300 Mega with a flame ionisation detector and fitted with a CP-Sil 5CB column (50 m) using H₂ as carrier gas. The temperature programme was 40–200°C (10°C min⁻¹) to 300°C (2°C min⁻¹), hold 30 min. Data acquisition was performed using a

Xchrom data system. Relative abundances were quantified by peak area measurements; concentrations were obtained using peak areas relative to that of the internal standard. GC–MS analysis was performed as described previously (Talbot et al., 1999a). Sterols were assigned by comparison of their mass spectra with standards and literature data.

3. Results

3.1. Chlorin distributions

3.1.1. *P. micans* experiment

The chlorin distribution (Fig. 1a) was dominated (see also Table 1) by both C-13² epimers of phaeophytin *a* (peaks 1 and 1', **IIa**) and their mono-oxygenated allomers, 13²-hydroxyphaeophytin *a* (peaks 2 and 2', **IIb**). There was no evidence of chl *a* (**IIIa**), indicating that the culture had reached stationary phase and was undergoing senescence (cf. Talbot et al., 1999a). Peak 3 has a similar electronic spectrum to chl *a* but mass spectral data indicate a possible oxygenated chl *a*-like component. Also present were two chl *c*-like components (peaks 4 and 5), detected only from electronic spectral data. A number of carotenoids were present which were not investigated further. The distribution in the algal control (Fig. 1b) was qualitatively similar except for the addition of peak 6. The electronic spectrum is similar to that of chl *a* but the mass spectrum again suggests an

oxygenated component, possibly 13²-hydroxychl *a* (**IIIb**), co-eluting with a non-absorbing component.

The fresh pellets (Fig. 1c) were dominated by pyrophaeophytin *a* (peak 7, **IVa**), with both C-13² epimers of phaeophytin *a* (1 and 1') and hydroxyphaeophytin *a* (2 and 2') as well as 15¹-hydroxyphaeophytin *a* lactone (8, **V**) present. Also present, co-eluting with a carotenoid, was pyrophaeophorbide *a* (9, **IVb**). Other minor components were an unknown phaeophytin *a*-like component (peak 10; tentatively proposed previously to be either mesopyrophaeophytin *a* [**IVc**] or pyrophaeophorbide *a* dihydrophytyl ester [**VIII**]; cf. Talbot et al., 1999a), purpurin-18-phytyl ester (11, **VI**) and 13²-oxopyrophaeophytin *a* (peak 12, **VII**; cf. Talbot et al., 1999a). A suite of six peaks (a–f) was apparent in the SCE region (see below).

After 8 days ageing, the distribution (Fig. 1d) was again dominated by pyrophaeophytin *a* (7) and pyrophaeophorbide *a* (9), the latter again co eluting with a carotenoid. Phaeophytin *a* (1 and 1'), hydroxyphaeophytin *a* (2 and 2'), purpurin-18-phytyl ester (11), and peak 10 were also present in trace abundance. The SCEs were in greater relative abundance than in the fresh pellets and after 29 days they dominated (Fig. 1e). Pyrophaeophorbide *a* (9), phaeophytin *a* (1 and 1'), purpurin-18-phytyl ester (11) and peak 10 were again present as minor components.

The sterilised pellet distribution was very similar to that of the fresh pellets except for the absence of pyrophaeophorbide *a* (peak 9). Given that this component

Table 1
Chlorin assignments and structures

Peak ^a	MH ⁺	Other ions	λ _{max} (nm)	Assignment	Structure
1	871	839, 813, 593, 561, 533, 517	407, 506, 533, 602, 665	Phaeophytin <i>a</i>	IIa
1'	871	839, 813, 593, 561, 533, 517	410, 506, 533, 602, 665	Phaeophytin <i>a</i> epimer	IIa
2	887	609, 591, 559	410, 503, 533, 611, 665	13 ² -Hydroxyphaeophytin <i>a</i>	IIb
2'	887	609, 591, 559	410, 503, 533, 611, 665	13 ² -Hydroxyphaeophytin <i>a</i> epimer	IIb
3	n.o. ^b	602, 584, 566, 554, 538	431, 587, 627, 666	Chlorophyll <i>a</i> -like	
4	n.o.		444, 545, 580, 630	Chlorophyll <i>c</i> -like	
5	n.o.		449, 580, 632	Chlorophyll <i>c</i> -like	
6	n.o.	631, 613, 582, 564, 553, 522, 495, 439	428, 528, 572, 615, 662	Chlorophyll <i>a</i> -like	
7	813	535	410, 470, 506, 536, 608, 665	Pyrophaeophytin <i>a</i>	IVa
8	903	625, 565	399, 495, 527, 609, 666	15 ¹ -Hydroxyphaeophytin <i>a</i> lactone	V
9	535		410, 506, 536, 608, 665	Pyrophaeophorbide <i>a</i>	IVb
10	815		410, 506, 536, 608, 665	Mesopyrophaeophytin <i>a</i> or pyrophaeophorbide <i>a</i> dihydrophytyl ester ^c	IVc VIII
11	843	565	407, 542, 584, 644, 695	Purpurin-18-phytyl ester	VI
12	827	549, 521, 503	389, 419, 512, 629, 674	13 ² -Oxopyrophaeophorbide <i>a</i>	VII
13	533	515, 505, 489	410, 506, 536, 608, 665	13 ² -Hydroxychlorophyllone <i>a</i>	IX
13'	533	515, 505, 489	410, 506, 536, 608, 665	13 ² -Hydroxychlorophyllone <i>a</i> epimer	IX
14	517		356, 422, 623, 683	Cyclophaeophorbide <i>a</i> enol	X

^a Refer to Figs. 1, 2 and 4.

^b n.o., not observed.

^c Assignment tentative.

Fig. 1. HPLC chromatograms (400 nm) from copepod grazing on *P. micans* (* = carotenoid; IS = internal standard). Peak identifications are given in Tables 1 and 2.

was the only carboxylic acid in the unsterilised pellets it seems likely that the Hg^{2+} salt was formed.

Mass chromatography revealed the presence of a total of 7 SCE MH^+ ions consistent with C_{27} – C_{30} mono- and diunsaturated or saturated sterols esterified to pyropheophorbide *a* (**IVb**). Spectra of the individual peaks (a–f, cf. Fig. 1; Table 2) all showed the expected fragment at m/z 535 corresponding to the pyropheophorbide *a* macrocycle. Peaks a–d and f each contained a single MH^+ ion and peak e contained two MH^+ ions with the m/z 919 component eluting just prior to the MH^+ m/z 945 component.

3.1.2. A tamarensis experiment

The chlorins in this culture (Fig. 2a) were dominated by both C-13² epimers of phaeophytin *a* (1 and 1') and hydroxyphaeophytin *a* (2 and 2'), again indicating that the alga was undergoing senescence. Surprisingly, pyropheophytin *a* (7, **IVa**) was also present, providing further evidence for senescence (Spooner et al., 1994). There were also a number of unknown chl-related components (unlabelled) and non-absorbing components present.

The control (Fig. 2b) was similar although there were now a number of other chl *a* transformation products present, including both C-13² epimers of hydroxychlorophyllone (chlorophyllone **IX**; peaks 13 and 13'; e.g. Sakata et al., 1990; Watanabe et al., 1993). The

Table 2
SCE sterol assignments and structures

Peak ^a	SCE MH^+	Esterifying sterol	Structure
b	903	Cholest-5-en-3 β -ol	A1
a	915	24-Methylcholesta-5,22E-dien-3 β -ol	A4
e	919	4 α -Methyl-5 α -cholestan-3 β -ol	C1
c	929	23,24-Dimethylcholesta-5,22E-dien-3 β -ol	A5
d	931	24-Ethylcholesta-5,22E-dien-3 β -ol	A6
		4 α ,24-Dimethylcholest-22E-en-3 β -ol	C4
e	945	24-Ethylcholest-5-en-3 β -ol	A7
		4 α ,23,24-Trimethyl-5 α -cholest-22E-en-3 β -ol	C5
f	947	4 α ,23,24-Trimethyl-5 α -cholestan-3 β -ol	C8

^a See Figs. 1 and 2.

Fig. 2. LC-MS base peak chromatograms from copepod grazing on *A. tamarensis*.

spectrum of peak 14 consists of a single ion (MH^+) at m/z 517 (Table 1), consistent with 13²,17³-cyclophaeophorbide *a* enol (**X**; e.g. Ocampo et al., 1999). This assignment was confirmed by comparison of the electronic spectrum with that given by Ocampo et al. (1999). Also present were 13²-oxopyropheophytin *a* (12, **VII**) and purpurin-18-phytyl ester (11, **VI**). The pellet distribution (Fig. 2c) was similar but with an increased proportion of pyropheophytin *a* (7) relative to phaeophytin *a*, reinforcing the common production of this component during grazing (e.g. Head and Harris, 1992). There was no indication of peak 14 (13²,17³-cyclophaeophorbide *a* enol) although both C-13² epimers of chlorophyllone (13 and 13'), 13²-oxopyropheophytin *a* (12) and purpurin-18-phytyl ester (11) were still present.

Mass chromatography revealed two SCE peaks (b and e) and the spectra (Table 2) showed the expected fragment at m/z 535. Peak b with MH^+ at m/z 903 indicates a mono-unsaturated esterifying C_{27} sterol and peak e with MH^+ at m/z 945 a mono-unsaturated C_{30} sterol. The pellets aged for 30 days showed essentially the same distribution.

3.2. Sterols

3.2.1. *P. micans* experiment

The starved animal distribution (Fig. 3a and Table 3) was dominated by cholesterol (peak 2, **A1**) along with cholesta-5,24-dien-3 β -ol (4, **A2**) and cholesta-5,22E-dien-3 β -ol (1, **A3**) as expected; 5 α -cholestan-3 β -ol (3, **B1**) was present in trace abundance. The two C_{27} diunsaturated sterols were not apparent as esters in the SCE mass chromatograms (m/z 901).

The algal distribution contained a complex mixture of 10 sterols (Fig. 3b and Table 3). This is a more complex distribution than that observed by Piretti et al. (1997) who reported only two major sterols, 4-methyl-24-ethylcholestan-3 β -ol (48.1%) and 4,23,24-trimethyl-5 α -cholest-22E-en-3 β -ol (54.6%) and minor contributions from 4 α ,24-dimethyl-5 α -cholest-22E-en-3 β -ol (6.3%) and 4-methyl-5 α -cholestan-3 β -ol (trace abundance). It is possible that the condition of the cells, i.e. that they were undergoing senescence, may have resulted in the increased number of components observed here. In the present study the 4-desmethyl components were dominated by cholesterol (2, **A1**), commonly found in dinoflagellates and often the major sterol (e.g. Volkman, 1986). Other significant 4-desmethyl sterols were 24-methylcholesta-5,22E-dien-3 β -ol (5, **A4**), 23,24-dimethylcholesta-5,22E-dien-3 β -ol (7, **A5**), 24-ethylcholesta-5,22E-dien-3 β -ol (8, **A6**), with 24-ethylcholest-5-en-3 β -ol (10, **A7**) and 24-ethyl-5 α -cholestan-3 β -ol (11, **B7**) in trace abundance. As expected, the 4-methyl sterols were dominated by dinosterol (12, **C5**) and 4 α ,23,24-trimethyl-5 α -cholestan-3 β -ol (dinostanol; 13, **C8**), with 4 α ,24-dimethyl-5 α -cholest-22E-en-3 β -ol (9, **C4**) and 4-methyl-5 α -cholestan-3 β -ol (6, **C1**) as minor components.

Fig. 3. Partial RIC traces (*P. micans* experiment) of free sterols in (a) starved animals, (b) culture and (c) faecal pellets (as TMSi ethers; unlabelled peaks are non-sterol components).

Table 3
Free sterol assignments and structures

Peak no. ^a	Sterol	Abbreviation ^b	Structure
1	Cholesta-5,22E-dien-3 β -ol	$C_{27} \Delta^{5,22}$	A3
2	Cholest-5-en-3 β -ol	$C_{27} \Delta^5$	A1
3	5 α -Cholestan-3 β -ol	$C_{27} \Delta^0$	B1
4	cholesta-5,24-dien-3 β -ol	$C_{27} \Delta^{5,24}$	A2
5	24-Methylcholesta-5,22E-dien-3 β -ol	$C_{28} \Delta^{5,22}$	A4
6	4 α -Methyl-5 α -cholestan-3 β -ol	4-Me $C_{28} \Delta^0$	C1
7	23,24-Dimethylcholesta-5,22E-dien-3 β -ol	23,24-Me $C_{29} \Delta^{5,22}$	A5
8	24-Ethylcholest-5,22E-dien-3 β -ol	24-Et $C_{29} \Delta^{5,22}$	A6
9	4 α ,24-Dimethyl-5 α -cholest-22E-en-3 β -ol	4,24-Me $C_{29} \Delta^{22}$	C4
10	24-Ethylcholest-5-en-3 β -ol	$C_{29} \Delta^5$	A7
11	24-Ethyl-5 α -cholestan-3 β -ol	$C_{29} \Delta^0$	B7
12	4 α ,23,24-Trimethyl-5 α -cholest-22E-en-3 β -ol	4,23,24-Me $C_{30} \Delta^{22}$	C5
13	4 α ,23,24-Trimethyl-5 α -cholestan-3 β -ol	4,23,24-Me $C_{30} \Delta^0$	C8

^a See Fig. 3.

^b See Figs. 4–6.

The pellet distribution (Fig. 3c) was similar and contained all 10 algal sterols, as well as cholesta-5,22E-dien-3 β -ol (**A3**) and 5 α -cholestan-3 β -ol (**B1**) which were present in the animal (Fig. 3a). There was no indication of cholesta-5,24-dien-3 β -ol (peak 4, **A2**) which was present in the animal only in trace abundance. A non-sterol component co-eluted with peak 9 so the relative abundance of 4 α ,24-dimethyl-5 α -cholest-22E-en-3 β -ol (9, **C4**) in the pellets is overestimated (see below).

Comparison of the SCE and free C₂₉ mono- and diunsaturated sterols is difficult. The culture contained 2 C₂₉ diunsaturated sterols (Table 3; **A5**, **A6**), corresponding to SCE peak c (MH⁺ at *m/z* 929; Table 2). Likewise the 2 C₂₉ monounsaturated sterols in the culture (Table 3; **C4**, **A7**) correspond to SCE peak d (MH⁺ at *m/z* 931; Table 2). There was insufficient material to isolate the SCE fraction and obtain the sterols by hydrolysis or LiAlH₄ reduction for analysis by GC–MS, so the contribution of each of the isomeric sterols to SCE peaks c and d could not be distinguished (Fig. 4). All other SCEs corresponded to a single available sterol and were assigned accordingly (Table 2); there was no indication of an SCE with MH⁺ at *m/z* 933 corresponding to the minor C₂₉ stanol component (peak 11 in Fig. 3). Comparison of the SCE and culture distributions reveals a decrease in the abundance of 4-methyl sterols in the SCEs relative to the culture (Fig. 4; for abbreviations see Table 3).

3.2.2. *A. tamarensis* experiment

The culture contained only two sterols (cholesterol [**A1**] and dinosterol [**C5**]), which is consistent with the SCE results. The presence of only the single 4-methyl component, dinosterol, is consistent with results for the same species reported by Piretti et al. (1997). Comparison of the abundance of the free and SCE sterols (based on MH⁺ peak areas) again showed a decrease in the abundance of the 4-methyl sterol (from 37 to 22% of the

sterols). It should be noted that there was probably a small contribution of cholesterol from the animal to the SCE although this is again thought to be negligible (cf. *P. micans* and Talbot et al., 1999a) as there was no indication in the SCEs of the two diunsaturated C₂₇ sterols typical of the copepod and previously found to be incorporated into SCEs during grazing on a diatom and a prasinophyte (cf. Talbot et al., 1999a, b).

3.3. Ageing studies

The relative abundance of the free sterols in the pellets over the first 8 days (Fig. 5; for abbreviations see Table 3) showed little change in the major algal sterols (cholesterol, 24-methylcholesta-5,22E-dien-3 β -ol, 23,24-dimethylcholesta-5,22E-dien-3 β -ol, 4 α ,24-dimethyl-5 α -cholest-22E-en-3 β -ol, dinosterol and dinostanol). After 29 days there was significant change, with the two major 4-methyl sterols in greater abundance, as was 23,24-dimethylcholesta-5,22E-dien-3 β -ol. Unfortunately the behaviour of the third most abundant 4-methyl sterol

Fig. 5. Relative distribution of free sterols in faecal pellets with ageing (*P. micans* experiment). For abbreviations see Table 3 (NB normalised to C₂₇ Δ^5 to show 4-Me effect).

Fig. 4. Comparison of SCE and culture free sterol distributions (*P. micans* experiment). For abbreviations see Table 3 (NB normalised to C₂₇ Δ^5 to show 4-Me effect).

Fig. 6. Faecal pellet SCE relative abundance with ageing (based on peak areas in HPLC 400 nm chromatogram; *P. micans* experiment). For abbreviations see Table 3 (NB normalised to C₂₇ Δ^5 as most abundant SCE sterol).

(4,24-dimethyl-5 α -cholest-22E-en-3 β -ol) could not be individually determined as it co-eluted (Fig. 3) with a non-sterol component. The distribution of free sterols in the sterilised pellets showed, however, a good correlation with those in the fresh pellets (Fig. 5).

The abundance of free sterol per pellet showed a trend of continual reduction (cf. Harvey et al., 1987) over the 29 day period (ca. 8 to 2 ng) but the abundance in the sterilised pellets was similar to that in the fresh pellets. The reduction in the sterols over the three stages of ageing was greater than that in the SCEs (see below), with the free sterols being significantly more abundant (ca. 1 order of magnitude) in the fresh pellets and the ratio falling to ca. 3 after 29 days. This indicates that the SCEs were significantly more stable under these conditions.

The SCE distribution at the three stages of ageing and in the sterilised pellets in the *P. micans* case showed no significant change over the 29 day period (Fig. 6, for abbreviations see Table 2; cf. also Fig. 1). The same was true for *A. tamar-ense*. The SCE contribution to the total chlorins showed, however, a rise as observed previously in a diatom study (Talbot et al., 1999a), increasing from ca. 17 to 31% (8 days) and 59% (29 days). Their abundance in the sterilised pellets was higher than in the fresh pellets (ca. 30 vs. 17%), presumably due to the absence of pyropheophorbide *a* from the total chlorins in the sterilised pellets. The abundance of SCEs per pellet (calculated relative to the internal standard as pyropheophytin *a* equivalents) showed an initial drop from ca. 0.8 to ca. 0.4 ng, surprisingly followed by a rise to ca. 0.6 ng for the 29 day sample. The latter seems highly unlikely given the results from the sterol concentrations and those from a similar study with a diatom (Talbot et al., 1999a). It appears, therefore, that the apparent rise was due to inaccuracy in the particular method used to separate the pellet aliquots in this experiment (cf. Talbot et al. 1999a, and see below) and that the drop in free sterol concentration after 29 days was actually greater than that observed.

4. Discussion

4.1. SCE formation

The production of SCEs has now been demonstrated during grazing on two members (*P. micans* and *A. tamar-ense*) of a third major algal division, the Dinophyta, having been observed previously in members of the Bacillariophyta and the Chlorophyta (cf. Talbot et al., 1999a, b).

The *P. micans* experiment revealed incorporation of all of the major algal sterols into the SCE fraction, but with significant discrimination against the 4-methyl sterols (Fig. 4) which comprised 72% of the available free sterols but only 28% of the SCE sterols (excluding C₂₉ mono- and diunsaturated components for reasons

given above). A similar effect occurred with *A. tamar-ense*. We suggest that this effect may be a result of steric hindrance involving the 4-methyl group, which slows the rate of the esterification process, leading to a lower proportion of 4-methyl components in the SCE fraction relative to the sterols of the substrate. Previous studies of sedimentary samples have found 4-methyl components to be significantly more abundant in the free sterol fraction than in the SCE fraction and the behaviour has been ascribed to a greater resistance to biodegradation of free 4-methyl sterols relative to free 4-desmethyl sterols (see Introduction). Indeed, preferential biodegradation of the free 4-desmethyl sterols was also seen clearly in the pellet ageing experiments, whereas the free sterol distribution did not change in the sterilised aged pellets (Fig. 5). Preferential 4-desmethyl sterol biodegradation indicates that use of free sedimentary sterols as indicators of phytoplankton community structure could lead to an overestimation of the input from dinoflagellates (and other phytoplankton species containing 4-methyl sterols). The present laboratory study suggests that the lower abundance of 4-methyl sterols in SCE fractions could also arise from a second process, i.e. discrimination against their incorporation into SCEs during herbivory.

Two of the three animal sterols (cholesta-5,22E-dien-3 β -ol [A3] and 5 α -cholestan-3 β -ol [B1]) which were not present in *P. micans* were present in the pellets (Figs. 3 and 5), although the third (cholesta-5,24-dien-3 β -ol, A2) was absent. This agrees with the observations of Harvey et al. (1987) who did not find this component in faecal pellets produced by *C. helgolandicus* fed a dinoflagellate at three different concentrations, presumably as it was retained for conversion to cholesterol (Goad, 1978, 1981).

4.2. SCE stability

As observed for a diatom (Talbot et al., 1999a), the abundance of the SCEs relative to total chlorins increased significantly over the 29 day ageing in the *P. micans* experiment; this provides additional evidence for the idea that SCEs are significant pigment components in aquatic sedimentary environments due to enhanced stability relative to other chlorins (King and Repeta, 1991, 1994; Talbot et al., 1999a).

Because of difficulties with the particular method used in this case to separate the pellets from the *P. micans* experiment into equal portions (dilution as opposed to manual counting in our earlier diatom experiment; Talbot et al., 1999a) there was inaccuracy in the estimation of the SCE abundance in the pellets after 29 days; however, from the marked drop after 8 days, taken with the results of the earlier study and the results for the free sterols, it does appear that significant degradation did occur over the 29 days period. The similarity in SCE concentration in the fresh and

sterilised pellets after 29 days ageing suggests that the degradation is a result of the effect of the bacterial community in the pellets rather than a chemical process, although the process remains to be determined. Nevertheless, the sterol distribution within the SCEs remained constant. Hence, although SCE degradation occurs, esterification to pyropheophorbide *a* then protects both 4-methyl and 4-desmethyl sterol distributions from a selective effect. The rapid decrease in the abundance of free sterols relative to the SCEs during pellet ageing indicates that SCEs are more stable than the free sterols. This, taken with the consistency in the SCE sterol distribution, provides laboratory evidence in favour of the proposal (King and Repeta, 1994) that sedimentary SCE sterol distributions are more robust than the corresponding free sterol distributions.

The pellet free sterol distribution showed little alteration over the initial 8 days ageing period although there was a drop in abundance of the major desmethyl sterols relative to the two most abundant 4-methyl sterols after 29 days. This provides further evidence for the need for caution in the use of free sedimentary 4-methyl sterols to estimate relative inputs from dinoflagellates.

4.3. Other chlorins

The chlorin signatures in the *A. tamarensis* experiment revealed some unexpected components in the culture, control and pellets (Fig. 2). The absence of chl *a* and presence of pyropheophytin *a* (IVa) in the culture indicates that it had started to undergo senescence prior to feeding since this component has been observed previously in senescent systems (Spooner et al., 1994; Louda et al., 1998). The control contained both C-13² epimers of chlorophyllone (IX; e.g. Sakata et al., 1990; Harris et al., 1995). This rearranged bicyclic chlorin has previously only been associated with diatoms (e.g. Watanabe et al., 1993), the only exception being its presence as a minor component in the haptophyte *I. galbana* which was incorrectly assigned as a diatom (Sakata et al., 1994). In our earlier diatom experiment (Talbot et al., 1999a) it was not found in the culture, control or fresh pellets. It was suggested that it was only detected in aged pellets as a result of greater stability relative to other chlorins, because it had been reported to occur in diatoms (Sakata, et al. 1990, 1994). Its presence in the *A. tamarensis* control and in the pellets further supports the idea that it is a product of algal enzyme activity.

Component X, 13²,17³-cyclophaeophorbide *a* enol, originally detected in a sponge (Karuso et al., 1986), has not been reported previously in marine algae. It has been found, however, in sediment trap material and as a major chlorin in a number of sediments (Ocampo et al., 1999). It is considered to be a precursor of other sedi-

mentary chlorins and porphyrins containing the seven + five exocyclic ring system such as chlorophyllone, so it is perhaps not surprising that both components were present in the algal control. In the *P. micans* experiment the oxidation product, 13²-oxopyropheophytin *a* (VII), was only found in the pellets as in our previous diatom study (Talbot et al., 1999a). Its presence in the *A. tamarensis* control, as well as in the pellets, provides evidence that it is, like chlorophyllone, a product of algal enzyme activity.

5. Conclusions

The formation of SCEs during grazing of the copepod *C. helgolandicus* on two dinoflagellates has been shown. Both 4-desmethyl and 4-methyl sterols were incorporated into the SCEs but there was significant discrimination against the uptake of the 4-methyl components in both cases. This behaviour is thought to be a result of steric hindrance due to the presence of the 4-methyl group and provides a possible second mechanism by which the relative abundance in sediments of 4-methyl sterols is lower in the SCE fraction than in the free sterol fraction. This means that, whereas using the abundance of free 4-methyl sterols relative to their desmethyl counterparts can overestimate the contribution from dinoflagellates, use of SCE 4-methyl sterols might underestimate the dinoflagellate contribution. On the other hand, once esterified, the SCE sterol distribution was more stable than the free sterol distribution during pellet ageing, so our results provide further evidence that sedimentary SCE sterols provide a better indicator of phytoplankton community structure than the corresponding free sedimentary sterols.

A number of chl transformation products, previously only associated with diatoms, have been observed in a senescent dinoflagellate culture (*A. tamarensis*) and in faecal pellets derived therefrom, one of which was also present in pellets derived from *P. micans*. These findings reveal a new source for such components, some of which have been found to be amongst the most abundant chlorins in certain sedimentary environments.

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Appendix

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