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Trophic upgrading of food quality by protozoans enhancing copepod growth: role of essential lipids

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Abstract Protozoa are known for their intermediary trophic role in transferring organic matter from small size planktonic particles to mesozooplankton. This study concentrates on the possible addition of biochemical value during this transfer, by new production of compounds that are essential in copepod food. In laboratory experiments, copepods could not be raised on a diet of the chlorophycean *Dunaliella* sp., though they readily consumed this alga. *Dunaliella* sp. contained all essential amino acids, but was deficient in highly unsaturated fatty acids and in sterols. In contrast to copepods, the heterotrophic dinoflagellate *Oxyrrhis marina* grew well on *Dunaliella* sp., producing significant amounts of the long-chain fatty acids docosahexaenoic acid and eicosapentaenoic acid, in addition to cholesterol and brassicasterol. Using this *O. marina* grown on *Dunaliella* sp. to feed *Temora longicornis* and *Pseudocalanus elongatus*, both copepod species rapidly developed from young nauplius larvae to maturity on the dinoflagellate diet. Hence, in this experimental food-chain the inadequate chlorophycean food was biochemically upgraded by the protozoan to high-quality copepod food. The results indicate that highly unsaturated fatty acids and/or sterols are essential compounds, which can be produced by protozoans. Due to their intermediate size, the mechanism of trophic upgrading by protozoans may bridge the gap of essential nutrients between the microbial loop and higher trophic levels.

Introduction

Generally, copepod growth or egg production is correlated with food availability, indicative of seasonal food limitation in the sea (e.g. Gifford and Dagg 1991; Klein Breteler and Schogt 1994; Hirst and Lampitt 1998). Zooplankton food, however, is often poorly defined by terms such as chlorophyll *a* or particulate organic carbon (POC) concentration (e.g. Claustre et al. 1989). Hence, differences of food quality often mask apparent relationships with food abundance (Donaghay 1985; Ambler 1986; Diel and Klein Breteler 1986; Kiørboe et al. 1988; Kleppel 1992; Jonasdottir et al. 1995). Clearly, there are many factors that affect the quality of copepod food in different ways. The size, edibility, repellence, and toxicity of food particles influence the grazing efficiency (Huntley 1988), whereas the biochemical composition (Kleppel 1993) and the connected assimilation efficiency (Mayzaud et al. 1998) of the food determine its nutritive value, which is reflected in the copepod's growth efficiency.

The nutritional value of food can not be assessed easily. Experimental studies have shown differences in the nutritional value of different microplanktonic taxa (Koski et al. 1998 and references therein). Comparing stoichiometric model predictions with experimental studies, Anderson and Hessen (1995) concluded that copepod nutrition is not determined by the elemental composition of the food only. They suggested that specific, essential food compounds play a significant role. In both freshwater and marine environments there are strong indications that the polyunsaturated fatty acids (PUFAs, with 20 or more C-atoms termed HUFAs) eicosapentaenoic acid (20:5ω3, EPA) and docosahexaenoic acid (22:6ω3, DHA) are such essential compounds that can limit zooplankton productivity (Jonasdottir et al. 1995; Müller-Navarra 1995). Laboratory observations show that sterols are of similar importance in copepod nutrition (Ederington et al. 1995). Typically, EPA occurs in significant amounts in diatoms, whereas DHA is abundant in dinoflagellates (Sargent et al. 1987 and references therein; Brown et al. 1997), including heterotrophic dinoflagellates (Harrington et al. 1970). Plant material is also a major source of sterols, although several groups of invertebrates, but

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not arthropods, have maintained the capacity to synthesise them (Dewey 1967; Goad 1981). Due to seasonal differences in the abundance of phytoplankton and protozoans, and since their lipid composition is also influenced by the physiological conditions (Sargent et al. 1987), the availability of essential lipids varies seasonally (Claustre et al. 1989; Mayzaud et al. 1989), thereby influencing zooplankton growth (Ahlgren et al. 1997).

Microzooplankton contribute significantly to the food of mesozooplankton, channelling energy from the microbial loop to higher trophic levels (see reviews by Sherr et al. 1986; Stoecker and Mc Dowell Capuzzo 1990; Gifford 1991; Gifford and Dagg 1991). Protozoans feed on a wide particle size spectrum (Capriulo et al. 1991), including small detritus, and pico- and nanoplankton. They may consume a significant proportion of the primary production (Capriulo et al. 1991), which partly becomes accessible to the copepods by consuming the intermediary protozoans. This has been called trophic repackaging by Gifford (1991). Although it may be an inefficient route of energy flow (Nagata et al. 1996), copepods may at least seasonally rely upon this energy source in most nearshore and oceanic environments (Stoecker and Mc Dowell Capuzzo 1990 and references therein). In addition to energy, protozoans transfer minerals, vitamins, amino acids, fatty acids and sterols from lower trophic levels, thus balancing any nutritional shortfalls of herbivory (Gifford and Dagg 1991). In this view protozoans are regarded as accumulators and redistributors of food. However, protozoans may also produce new compounds, such as essential lipids, which would mean that they are also upgrading the biochemical composition of food, rather than merely repackaging it.

Heterotrophic flagellates and ciliates are known for their high content of HUFAs and sterols (Dewey 1967; Harrington and Holz 1968; Harrington et al. 1970; Dikarev et al. 1982; Claustre et al. 1989; Stoecker and Mc Dowell Capuzzo 1990). In marine species little is known about the origin of these lipids. In bacteria and detritus, HUFAs and sterols are rare or absent (Phillips 1984; Stoecker and Mc Dowell Capuzzo 1990; Brown et al. 1996), thus repackaging of this food would not contribute to the richness of essential lipids found in marine protozoans. Hence, the main source of essential lipids present in protozoans must be sought in algae. Only one species of protozoan, the heterotrophic dinoflagellate *Crypthecodinium cohnii*, is known to de novo synthesise DHA (Harrington and Holz 1968; Barclay et al. 1994). Two other heterotrophic dinoflagellates, *Noctiluca scintillans* and *Gyrodinium lebourae*, can grow on simple dissolved compounds such as glucose, acetate and amino acids (Gaines and Elbrächter 1987), while *Oxyrrhis marina* requires specific lipids, such as quinones or sterols to grow (Droop 1966; Droop and Pennock 1971). Although this certainly shows some of the biosynthetic capacities of protozoans, their growth does not necessarily imply the production of HUFAs or sterols. At present, the scarce data available suggest that, except for *C. cohnii*, marine heterotrophic dinoflagellates and ciliates are not able to produce HUFAs or sterols (Ederington et al. 1995; Kleppel and Burkart 1995; Harvey et al. 1997). This would imply that they obtain them directly from their algal food, which is consistent with the idea of repackaging. However, considering the wide variety of synthetic capabilities among freshwater protozoans (Dewey 1967; Anderson 1988; Sleigh 1989), it seems probable that also other marine species may produce HUFAs and sterols by converting simple lipids from bacteria and detritus, in addition to the direct transfer from algae.

Lack of data on the biosynthetic capabilities of heterotrophic marine protozoans (Phillips 1984) is due to the fragility and specific food requirements of most protozoans, which allows culturing of only a few neritic species (Gaines and Elbrächter 1987). In the present study we used the heterotrophic dinoflagellate *Oxyrrhis marina* (Dujardin), which occurs in supra-tidal pools (Droop 1959) as well as in neritic surface waters (Tong 1997). We searched for essential nutritional compounds for copepods and tested the hypothesis that these compounds present in *O. marina* can be produced by this flagellate. Furthermore we compared concentrations of amino acids, fatty acids and sterols in *O. marina* with those in its algal food. To assess any improved food value during trophic transfer from alga to protozoan, we measured the rate of development of copepods grazing on the same algae and on the protozoan.

Materials and methods

Experiments

Using continuous cultures, the chlorophycean *Dunaliella* sp. and the cryptophycean *Rhodomonas* sp. were fed separately to continuous cultures of the protozoan *Oxyrrhis marina* (Dujardin). The two protozoan cultures, called Ox (d) and Ox (r), respectively, were fed separately to young stages of the copepods *Temora longicornis* (Müller) and *Pseudocalanus elongatus* (Boeck). Using *O. marina* as food, six and four experiments were done with *T. longicornis* and *P. elongatus*, respectively. Using algae as food for copepods, only two experiments were done with *Dunaliella* sp., since this merely was a repetition of a larger study using a variety of algae as food for *P. elongatus* (Koski et al. 1998). The fatty acid and the amino acid composition of algal and protozoan food were measured as described below.

The development time of the copepods was measured in glass bottles of 1.2 litres, which were rotating at 1 rpm using a rolling apparatus in a temperature-controlled room at 15 °C under strongly dimmed natural daylight conditions. At the start, each bottle contained about 400 to 800 (depending on stage) young larvae of *Temora longicornis* or *Pseudocalanus elongatus*. They were taken from a brood stock, pre-washed and incubated in double-filtered (2 μm) seawater. The stage of the larvae varied from Nauplius I to Copepodite I, but within experiments they were in a narrow cohort of mainly two to four stages. Incubation continued until most copepods were mature or dead. Food was supplied from the continuous cultures of algae or protozoans at a concentration of ≥300 μg C l^{-1}. Every day 90% of the food medium was siphoned out, using reversed flow filtration with 50 μm gauze, and replaced by new medium with food. Two times per week, the food concentration was measured using an Elzone electronic particle

counter (Particle Data Inc.). Sampling of copepods started 1 d after incubation to allow adaptation to the new food. This sampling occurred three times per week to determine the stage distribution, at the same time diluting the culture to keep the copepod biomass constant at a value equivalent to about 40 adult specimens l^{-1}. Stage duration was calculated from the median development time, estimated using a Gamma distribution function to describe the cumulative stage distribution as described by Klein Breteler et al. (1994).

Cultures

Copepods were obtained from brood stocks of *Temora longicornis* and *Pseudocalanus elongatus*, which were continuously cultured in the laboratory at 15 °C with surplus of food (> 300 μg C l^{-1}) as described by Klein Breteler and Gonzalez (1986, 1988). The food consisted of the cryptophyte *Rhodomonas* sp., the haptophyte *Isochrysis galbana* and the heterotrophic dinoflagellate *Oxyrrhis marina*. *O. marina* was held together with the copepods in the culture tanks at a concentration of about 1000 cells ml^{-1} (Klein Breteler and Laan 1993), equivalent to about 200 μg C l^{-1}.

The algae *Rhodomonas* sp., *Dunaliella* sp. and *Isochrysis galbana* were cultured at 15 °C in 1 to 3-litre chemostats, using sterile techniques, *f*/2 medium (Guillard 1975), constant air supply and a light intensity of ca. 150 μE m^{-2} s^{-1} in a 16 h light:8 h dark regime. The dilution rate was kept at about 0.2 d^{-1} for *Rhodomonas* sp. and *I. galbana*, and 0.5 d^{-1} for *Dunaliella* sp.

Oxyrrhis marina was cultured in a two-stage chemostat, fed with a supply of the *Rhodomonas* sp. or the *Dunaliella* sp. continuous culture, at a dilution rate of 0.18 and 0.25 d^{-1}, respectively. Cultures of *O. marina* were not kept sterile. They were aerated and stirred, and the flasks were wrapped in aluminium foil and replaced by clean ones every week. This dual system was run at about constant cell densities (Table 1) for at least 6 months.

Cell concentration and cell volume of the algae and the protozoan in samples from the continuous cultures were measured, using the Elzone particle counter. For amino acid analysis, samples were centrifuged (1100 ×*g*) and stored at −50 °C until analysis. For determination of carbon and lipid content, samples were filtered on combusted Whatman GF/F filters and stored under N_2 at −50 °C. The carbon content was measured using a Carlo Elba CHN analyser.

Lipid analysis

Samples of food algae and flagellates were ultrasonically treated for 2 min at room temperature and extracted in 6 ml methanol [MeOH (3×)], dichloromethane (DCM)/MeOH [1:1, v/v (3×)] and DCM (3×) to obtain the total lipid extract. Extracts of *Dunaliella* sp. were also saponified by reflux (1 h) with 1 *N* KOH/MeOH solution, to yield possible esterified lipids. After acidifying the solution to pH 3, double-distilled water, MeOH and DCM were added in a separatory funnel. The DCM fraction was collected, including double washing of the water/MeOH fraction with DCM. Total lipid extracts and saponified extracts were methylated with diazomethane in diethylether and eluted over a silica column with ethyl acetate. The extracts were subsequently silylated by adding 25 μl Bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (BSTFA), 75 μl pyridine and heating the mixture at 60 °C for 30 min. As internal standard 2,3dimethyl-5(1,1-dideutero-hexadecyl)thiophene was used to quantify the amount of fatty acids.

Extracted lipids were analysed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). Gas chromatography was carried out on a Hewlett-Packard 6890 series instrument equipped with an on-column injection port. A CP Sil-5 (0.12 μm film thickness, 25 m × 0.32 mm) column was used with helium as a carrier gas. Components were detected by a flame ionisation detector (FID). Samples were dissolved in ethyl acetate and injected at 70 °C. Subsequently the oven was programmed at 20 °C min^{-1} to 130 °C and then at 4 °C min^{-1} to 200 °C and finally at 8 °C min^{-1} to 320 °C, which was held isothermally (45 min). GC/MS was carried out on a Hewlett-Packard 5890 gas chromatograph interfaced with a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range of m/z 50 to 800 and a cycle time of 1.8 s (resolution 1000). The gas chromatograph was equipped with a fused silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 (film thickness 0.12 μm). Helium was used as carrier gas. The samples were injected at 60 °C and subsequently the oven was programmed at 20 °C min^{-1} to 130 °C and then at 4 °C min^{-1} to 320 °C, which was held isothermally (45 min). Double-bond positions were determined by comparing retention times of the extracted compounds with those of PUFA No. 1 and No. 2 standard mixtures (Matreya, Inc.).

Amino acid analysis

Samples of algae were vacuum dried, acid hydrolysed with 6 *N* HCL at 112 °C for 24 h and analysed using the Waters AccQ.Tag method with AccQ.Tag column, pre-column derivatization with 6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate (AQC) and HPLC technology (Waters Associates, 600 E gradient module with system controller and a Waters 470 scanning fluorescence detector), which allows the assessment of 17 different amino acids.

Results

High densities of *Rhodomonas* sp. and *Dunaliella* sp. were obtained in the chemostats. The cell diameter of these algae was quite similar, viz. 5 to 10 and 4 to 9 μm, respectively (Table 1). Using the outflow from these cultures as the inflow for the continuous culture of *Oxyrrhis marina*, a rather constant *O. marina* density was maintained. The cell size of *O. marina* was about the same (ca. 9 to 20 μm) with both species of algae used as food, but the abundance of *O. marina* when fed with *Rhodomonas*

Table 1 *Rhodomonas* sp., *Dunaliella* sp., *Oxyrrhis marina*. Characteristics of flagellates in chemostats. Mean values ± SE and number of measurements (n) of cell concentration (cells μl^{-1}), minimum and maximum cell size (equivalent spherical diameter, *ESD* in μm) and cell volume (μm^3) (Rho, *Rhodomonas* sp.; Duna, *Dunaliella* sp.; Ox (r), *O. marina* grown on *Rhodomonas* sp.; Ox (d), *O. marina* grown on *Dunaliella* sp.)

Species	Dilution rate (d^{-1})	Cell conc. (cells μl^{-1})	ESD (μm)		Cell volume (μm^3)	n
			Min.	Max.		
Rho	0.16	1750 ± 113	5.5 ± 0.0	10.2 ± 0.3	165 ± 3	11
Duna	0.50	1838 ± 204	4.3 ± 0.0	8.7 ± 0.1	98 ± 2	4
Ox (r)	0.18	129 ± 8	8.7 ± 0.2	22.6 ± 0.3	1589 ± 54	13
Ox (d)	0.25	60 ± 3	9.1 ± 0.1	19.7 ± 0.2	1145 ± 28	31

sp. was two times higher (129 cells μl^{-1}) than with *Dunaliella* sp. (60 cells μl^{-1}). The particle size spectra of the *O. marina* cultures did not reveal any remains of the food algae, which were virtually all consumed.

The amino acid distribution did not greatly vary among *Rhodomonas* sp., *Dunaliella* sp., and *Oxyrrhis marina* fed with *Rhodomonas* sp. (Table 2). Their distribution compares well with that of an "average" invertebrate (Phillips 1984), except methionine which was in relatively low abundance. Unfortunately, the amino acid composition of *O. marina* fed with *Dunaliella* sp. was not measured. However, since the amino acid composition of *Dunaliella* sp. and *Rhodomonas* sp. were quite similar, a similar composition may be expected in *O. marina* fed with *Dunaliella* sp.

The fatty acid distribution and the amount of sterols differed strongly among food species (Table 3). *Oxyrrhis marina* contained large amounts of DHA (22:6ω3), while EPA (20:5ω3), cholesterol (Δ5C27:1) and brassicasterol (Δ5,22C28:2) were also abundant. *Rhodomonas* sp. contained EPA, DHA, cholesterol and brassicasterol as well, hence these lipids in *O. marina* grown on *Rhodomonas* sp. may be derived from the food. In contrast, in *Dunaliella* sp. large quantities of 18:3(ω3?) fatty acids occurred. Unfortunately the double-bond positions could not be determined with certainty, but 18:3ω3 is the common type in *Dunaliella* spp. (e.g. Zhukova and Aizdaicher 1995). Importantly, *Dunaliella* sp. did not contain any HUFAs and only traces of Δ7 sterols. Saponified extract of *Dunaliella* sp. had a quite similar lipid distribution (not shown), also without any sign of HUFAs and only traces of Δ7 sterols. Hence, the HUFAs and Δ5 sterols in *O. marina* grown on *Dunaliella* sp. were not obtained from the algal food.

The stage duration of the larvae of *Temora longicornis* and *Pseudocalanus elongatus* was extremely long when *Dunaliella* sp. was offered as food (Table 4). The larvae remained in Naupliar Stage V or VI and died within 2 or 3 weeks. With the heterotrophic *Oxyrrhis marina* as food, the copepods rapidly developed from young nauplius larvae to maturity, irrespective of the species of alga (*Rhodomonas* sp. or *Dunaliella* sp.) used to grow *O. marina*. With both kinds of protozoan food the duration of the individual stages was around 2 d (Table 4). *T. longicornis* reached the adult stage in about 20 d, whereas *P. elongatus* needed about 23 d to become mature (Fig. 1). No experiments with *Rhodomonas* sp. were conducted. However, previous results (Klein Breteler et al. (1990) and comparisons with Koski et al. (1998) indicate that *O. marina* does not improve the *Rhodomonas* sp. diet for the two copepod species.

Table 2 *Dunaliella* sp., *Rhodomonas* sp. and *Oxyrrhis marina*. Amino acid distribution (% of total) and total concentration (mmol l^{-1} of cell volume). Species abbreviations as in Table 1

Amino acid	Species		
	Duna	Rho	Ox (r)
ASP	8.9	7.5	8.2
SER	7.0	8.5	10.5
GLU	13.3	9.9	13.8
GLY	12.7	14.2	14.8
HIS	1.7	2.2	2.4
ARG	5.2	6.6	4.7
THR	5.4	7.0	5.6
ALA	10.0	9.7	9.2
PRO	5.4	6.0	6.0
CYS	0.0	0.0	0.0
TYR	1.7	2.3	0.4
VAL1	6.4	5.8	5.4
MET	0.3	0.0	0.2
LYS	5.0	4.3	4.5
ILE	5.1	3.9	3.5
LEU	7.9	6.8	5.9
PHE	4.0	4.0	3.0
Total conc.	896	1696	1693

Table 3 *Rhodomonas* sp., *Dunaliella* sp. and *Oxyrrhis marina*. Distribution (% of total) and total amount (μg mg^{-1} C) of free fatty acids and of individual sterols (μg mg^{-1} C) in different species of flagellates. Trace amounts below detection limit (*tr*); uncertain identification (?) due to different GC-column used in analysis of the PUFA No. 2 standard mixture. Species abbreviations as in Table 1

	Species			
	Rho	Duna	Ox (r)	Ox (d)
Fatty acid (% of total FA)				
C14:0	9.3	0.4	6.3	1.3
C16:4	0.0	19.4	0.0	1.4
C16:3	0.0	3.5	0.0	0.0
C16:1ωx	0.0	3.7	0.0	0.4
C16:1ωx	1.4	0.4	0.0	0.4
C16:1ω7	1.3	0.8	1.0	0.7
C16:0	12.9	13.2	25.3	29.3
C18:x	2.3	0.0	0.0	0.0
C18:4ω3 + C18:3(ω6?)	16.1	0.0	2.8	1.6
C18:3ωx	0.0	4.8	0.0	0.0
C18:2ωx	0.7	0.0	0.0	0.0
C18:2ω6 + C18:3(ω3?)	20.3	46.6	4.5	16.2
C18:1ω9	13.4	3.6	4.3	1.9
C18:1ω7	6.8	3.2	3.2	2.2
C18:0	0.7	0.4	0.2	0.2
C20:5ω3	0.0	0.0	6.4	2.1
C20:5ω3 + C20:4	7.8	0.0	0.0	0.0
C22:6ω3	7.1	0.0	46.0	42.2
C22:4ω6	0.0	0.0	0.1	0.0
Total FA (μg mg^{-1} C)	46	119.7	91.8	85.7
Sterol (μg mg^{-1} C)				
Δ5,22C27:2				0.8
Δ5C27:1	0.1		1.9	2.3
Δ5,22C28:2	5.3		2.5	0.4
Δ5C28:1				0.1
Δ5,22C29:2				0.5
Δ7C28:1		tr		
Δ7C29:1		tr		

Discussion

The results from our experimental food chain clearly demonstrate the mechanism of trophic upgrading of food quality by an intermediary protozoan. It appears that copepods can not grow on *Dunaliella* sp., despite its suitable size and ready consumption (Koski et al. 1998).

Table 4 *Temora longicornis*, *Pseudocalanus elongatus*. Mean stage duration (d) of copepods fed with different food sources, abbreviated as in Table 1. Stages 3 to 6 (Nauplius III to VI) and 7 to 11 (Copepodite I to V). Number of observations (*n*) and standard deviation (*SD*)

Stage	Duna		Ox (d)			Ox (r)		
	Duration (d)	*n*	Duration (d)	*n*	SD	Duration (d)	*n*	SD
T. longicornis								
3	3.85	1	1.79	1		1.80	1	
4	8.09	1	1.36	1		1.32	1	
5			1.63	1		1.40	1	
6			1.69	3	0.39	2.12	2	0.98
7			2.17	4	0.34	1.75	2	0.02
8			1.75	6	0.16	1.90	3	0.08
9			1.68	6	0.13	1.69	3	0.16
10			2.20	6	0.47	1.85	3	0.16
11			2.07	6	0.42	1.97	3	0.25
P. elongatus								
4	5.84	1	3.50	1		3.33	1	
5	11.33	1	2.13	3	0.52	2.57	2	0.69
6			1.15	4	0.16	1.85	3	0.24
7			2.28	4	0.36	2.81	3	0.52
8			2.07	4	0.31	1.97	3	0.21
9			2.10	4	0.22	2.65	3	0.20
10			2.73	3	0.72	1.90	2	0.02
11			2.18	1		2.11	1	

Clearly, without adding food value by some essential compounds, simple repackaging of this food would not suffice to obtain suitable copepod food. A similar nutritional inadequacy of different species of *Dunaliella* was also reported in studies with ciliates (Verity and Villareal 1986), juvenile clams and oyster spat (Walne 1970; Langdon and Waldock 1981; Enright et al. 1986), and the copepods *Acartia clausi* (Støttrup and Jensen 1990) and *Pseudocalanus elongatus* (Koski et al. 1998). In contrast, the heterotrophic dinoflagellate *Oxyrrhis marina* grew well on *Dunaliella* sp. (Fuller 1990 and references therein), and thereby appeared to produce large amounts of HUFAs and sterols (present study). When feeding on this *O. marina*, both copepod species rapidly developed from larvae to the adult stage. Therefore, it seems that either EPA, DHA and/or sterols are the missing essential compounds in *Dunaliella* sp., which were added by the intermediary protozoan.

The present two-stage continuous cultures of algae and flagellates were typically agnotobiotic. Regarding possible influences of other micro-organisms, occasional microscopic observations revealed only bacteria, occurring in low numbers, in these cultures. Apart from a positive contribution as vitamins, other possibly confounding influences of bacteria are improbable. Bacteria do not contain sterols. They also have no HUFAs (Phillips 1984), except for some psychrophilic strains (Hamamoto et al. 1995; Nichols et al. 1996), due to homeoviscous adaptation to extreme temperatures and depths (Gibbs 1998). In addition, branched and odd-numbered fatty acids, characteristic of bacteria (Claustre et al. 1989), were absent in the samples from *Oxyrrhis marina* (Table 3). These markers would be maintained after consumption by protozoans (Harvey et al. 1997) and even after transfer into copepods (Ederington et al. 1995). Hence, there is little evidence for any supply of essential lipids by bacteria into the present experimental food chain, which supports our conclusion that they were produced by *O. marina*.

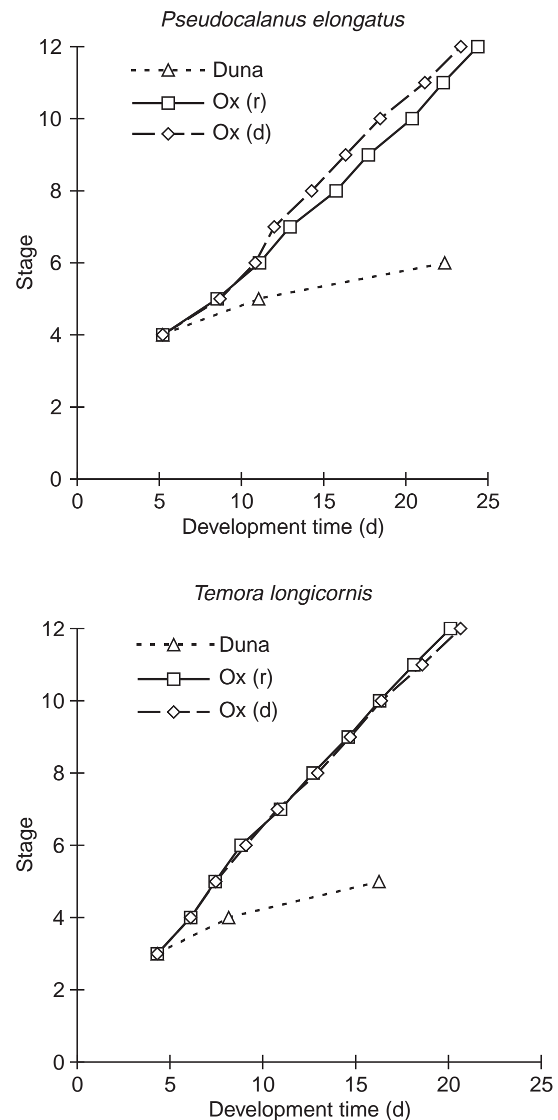

Fig. 1 *Pseudocalanus elongatus*, *Temora longicornis*. Cumulative development time (d) of copepod life stages at 15 °C using different food sources: *Dunaliella* sp. (*Duna*), *Oxyrrhis marina* grown on *Dunaliella* sp. [*Ox* (*d*)], or *O. marina* grown on *Rhodomonas* sp. [*Ox* (*r*)]. Egg (*0*), Nauplius I to VI (*1 to 6*), Copepodite I to V (*7 to 11*) and adult (*12*) stages. First data point obtained from Klein Breteler et al. (1994) at constant conditions of a mixed food used in stock cultures (cf. "Materials and methods")

Essential nutrients for copepods

There is no reason to assume a deficiency of amino acids in *Dunaliella* sp. or *Oxyrrhis marina*. Our results are in agreement with Cowie and Hedges (1992), Brown et al.

(1997) and Lourenço et al. (1998), who show only small differences in amino acid composition among algal species. Therefore, in our experiments, the poor copepod growth obtained with *Dunaliella* sp. can not be explained by the amount of different amino acids. We also measured (unpublished observations) quite similar amino acid compositions in the other algal species studied by Koski et al. (1998). Hence, the different rates of development, growth and egg production of *Pseudocalanus elongatus* observed by these authors also can not be explained by the amino acid composition of the algae. In contrast, using a histidine-deficient strain of *Isochrysis galbana*, Kleppel et al. (1998) showed that this amino acid is an essential nutrient for egg production of *Acartia tonsa*. These authors showed that *O. marina* feeding on this alga did contain high amounts of histidine, which apparently promoted egg production by the copepod and, therefore, may be another example of trophic upgrading of food quality.

HUFAs play an important role in a wide range of physiological processes in invertebrates and are suggested to control the growth of zooplankton in nature (Ahlgren et al. 1997; Brett and Müller-Navarra 1997). In various higher organisms, including cyclopoid and harpacticoid copepods, conversion from, e.g., 18:3ω3 fatty acids to EPA and further to DHA has been described (Ackman et al. 1968; Norsker and Støttrup 1994; Desvilettes et al. 1997). This conversion, however, is inefficient, and most animals grow better if direct sources of EPA and DHA are provided (Brett and Müller-Navarra 1997). Direct evidence for such a growth-stimulatory effect by HUFAs in zooplankton food was found by adding EPA and/or fish-oil rich in ω3 HUFA to the green algae *Scenedesmus* spp. or to the cyanobacterium *Synechococcus elongatus*, which were fed to different species of *Daphnia* (DeMott and Müller-Navarra 1997; Sundbom and Vrede 1997; Weers and Gulati 1997). However, HUFA-rich lipid emulsions used to supplement a diet of our strain of *Dunaliella* sp. did not enhance growth of *Pseudocalanus elongatus* (Koski et al. 1998), suggesting that HUFAs were not, or not the only, essential nutrients. Yeast-grown *Oxyrrhis marina*, not containing any HUFAs, supported high rates of egg production in *Acartia tonsa* (Kleppel et al. 1998). Hence, with the present knowledge, there is no direct proof that long-chain unsaturated fatty acids are essential for copepods.

Sterols are indispensable for several vital functions, such as maintenance of cellular architecture, membrane functioning, growth and reproduction (Ederington et al. 1995; Crockett 1998). Cholesterol is the dominant sterol in crustaceans (Goad 1981). It is a precursor of ecdysteroids, which are essential for moulting (Fingerman 1987). Since crustaceans are not capable of de novo synthesis of sterols (Goad 1981), they must be obtained from food. Isotope-ratio GC/MS analyses confirm that copepods derive their sterols from algal food, either directly or as precursor to produce other sterols (Grice et al. 1998). However, not all sterols seem to be useful: Δ7 sterols were released unchanged as faecal lipids by *Calanus helgolandicus*, in contrast to sterols with Δ5 and Δ5,7 nuclear unsaturation (Prahl et al. 1984). Therefore, the occurrence of traces of only Δ7 sterols and the absence of other sterols in our strain of *Dunaliella* sp. seems to indicate an essential dietary deficiency, which could explain why the copepods did not develop with *Dunaliella* sp. as food.

Production of lipids by protozoans

The present results reveal a significant capacity of HUFA production by *Oxyrrhis marina*. In addition to another heterotrophic dinoflagellate, *Crypthecodinium cohnii* (Barclay et al. 1994), autotrophic dinoflagellates synthesise HUFAs de novo, following a route that is possibly characteristic for this class of algae, as suggested from the stable carbon isotopic composition of two dinoflagellate species (Schouten et al. 1998). However, *O. marina* may have derived its HUFAs by converting other dietary fatty acids, such as the high amounts of 18:3(ω3?) and shorter, more saturated fatty acids present in *Dunaliella* sp. The high synthetic capability of this organism appears from its ability to grow in chemically defined media with only quinone and a sterol as essential compounds (Droop and Pennock 1971). However, this does not necessarily imply production of HUFAs. Kleppel and Burkart (1995) and Kleppel et al. (1998) only found HUFAs in *O. marina* when fed with the alga *Isochrysis galbana* (which already contained HUFAs), but not when fed with yeast. Yeasts have no HUFAs and a low content of PUFAs (Yongmanitchai and Ward 1989), which generally belong to the ω6 family in fungi (Phillips 1984). Thus they are quite different from the ω3 HUFAs in *O. marina*. For HUFA production, therefore, *O. marina* seems to need algae as food, possibly connected with the ω3 family of fatty acids they contain.

The present experiments give clear evidence of sterol production by *Oxyrrhis marina*. In *Dunaliella* sp. only traces of two Δ7 sterols were found. Although this kind of sterol supports growth of *O. marina* (Droop and Pennock 1971), the low amount observed seems insufficient for conversion into the sterols occurring in *O. marina*. Therefore, we believe that this heterotrophic flagellate is capable of synthesising cholesterol, brassicasterol and several other sterols, possibly by conversion of other lipids.

Ciliates are reported to grow poorly, or not at all, on *Dunaliella tertiolecta* (Heinbokel 1978; Verity and Villareal 1986), *D. primolecta* (Fuller 1990) and on cyanobacteria (Verity and Villareal 1986), although they all contain 18:3ω3 fatty acid (Sargent et al. 1987; Støttrup and Jensen 1990; Ahlgren et al. 1992). In three marine species of ciliates the lipid composition resembled that of their food, i.e. branched fatty acids and a lack of HUFAs and sterols if bacterivore, and the same PUFAs and sterols as present in the algal food in the

case of herbivory (Harvey et al. 1997). Therefore, marine ciliates do not seem to add value to the food consumed, apart from repackaging it.

In heterotrophic dinoflagellates, the capability of a few species to produce essential lipids may indicate a unique role for this group of protozoans. To quantify the ecological significance of this trophic upgrading of food quality in natural systems, much more information is required on the variety of species and the biochemical pathways involved. Around 50% of the dinoflagellates are obligate heterotrophs (Gaines and Elbrächter 1987) and many others are mixotrophs (Drebes 1974). The heterotrophic forms can occur abundantly in neritic waters, while in oceanic waters they may outnumber the ciliates (Fenchel 1987). Regarding the close relationship with autotrophic family members, it would not be surprising if many more of the truly or partly heterotrophic dinoflagellate species have maintained the capability to synthesise HUFAs and sterols de novo, or found ways to produce them by conversion from other lipids. Further research may elucidate the role of protozoans as a source of essential nutrients (Stoecker and Mc Dowell Capuzzo 1990; Gifford 1991) for zooplankton in oligotrophic oceans and in detritus-based food webs (Phillips 1984), and explain their unique position as intermediates between the microbial loop and higher trophic levels.

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References

Ackman RG, Tocher CS, McLachlan J (1968) Marine phytoplankter fatty acids. J Fish Res Bd Can 25: 1603–1620

Ahlgren G, Goedkoop W, Markensten H, Sonesten L, Boberg M (1997) Seasonal variations in food quality for pelagic and benthic invertebrates in Lake Erken – the role of fatty acids. Freshwat Biol 38: 555–570

Ahlgren G, Gustafsson I-B, Boberg M (1992) Fatty acid content and chemical composition of freshwater microalgae. J Phycol 28: 37–50

Ambler JW (1986) Effect of food quantity and quality on egg production of *Acartia tonsa* Dana from East Lagoon, Texas. Estuar cstl Shelf Sci 23: 183–196

Anderson OR (1988) Comparative protozoology. Springer, Berlin

Anderson TR, Hessen DO (1995) Carbon or nitrogen limitation in marine copepods? J Plankton Res 17: 317–331

Barclay WR, Meager KM, Abril JR (1994) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J appl Phycol 6: 123–129

Brett MT, Müller-Navarra DC (1997) The role of highly unsaturated fatty acids in aquatic food web processes. Freshwat Biol 38: 483–499

Brown MR, Barrett SM, Volkman JK, Nearhos SP, Nell JA, Allan GL (1996) Biochemical composition of new yeasts and bacteria evaluated as food for bivalve aquaculture. Aquaculture, Amsterdam 143: 341–360

Brown M, Jeffrey S, Volkman J, Dunstan G (1997) Nutritional properties of microalgae for mariculture. Aquaculture, Amsterdam 151: 315–331

Capriulo GM, Sherr EB, Sherr BF (1991) Trophic behaviour and related community feeding activities of heterotrophic marine protists. In: Reid PC, Turley CM, Burkill PH (eds) Protozoa and their role in marine processes. Springer, Berlin, pp 219–265

Claustre H, Marty JC, Cassiani L, Dagaut J (1989) Fatty acid dynamics in phytoplankton and microzooplankton communities during a spring bloom in the coastal Ligurian Sea: ecological implications. Mar microb Fd Webs 3: 51–66

Cowie GL, Hedges JJ (1992) Sources and reactivities of amino acids in a coastal marine environment. Limnol Oceanogr 37: 703–724

Crockett E (1998) Cholesterol function in plasma membranes from ectotherms: membrane-specific roles in adaptation to temperature. Am Zool 38: 291–304

DeMott WR, Müller-Navarra DC (1997) The importance of highly unsaturated fatty acids in zooplankton nutrition: evidence from experiments with *Daphnia*, a cyanobacterium and lipid emulsions. Freshwat Biol 38: 649–664

Desvilettes C, Bourdier G, Breton JC (1997) On the occurrence of a possible bioconversion of linolenic acid into docosahexaenoic acid by the copepod *Eucyclops serrulatus* fed on microalgae. J Plankton Res 19: 273–278

Dewey VC (1967) Lipid composition, nutrition and metabolism. In: Florkin M, Scheer BT (eds) Chemical zoology. Vol. I. Protozoa. Academic Press, New York, pp 161–274

Diel S, Klein Breteler WCM (1986) Growth and development of *Calanus* spp. (Copepoda) during spring phytoplankton succession in the North Sea. Mar Biol 91: 85–92

Dikarev VP, Svetashev VI, Vaskovsky VE (1982) *Noctiluca miliaris*– one more protozoan with unusual lipid composition. Comp Biochem Physiol 72B: 137–140

Donaghay PL (1985) An experimental test of the relative significance of food quality and past feeding history to limitation of egg production of the estuarine copepod *Acartia tonsa*. Arch Hydrobiol (Beih Ergebn Limnol) 21: 235–245

Drebes G (1974) Marines Phytoplankton. Thieme, Stuttgart

Droop MR (1959) Water-soluble factors in the nutrition of *Oxyrrhis marina*. J mar biol Ass UK 38: 605–620

Droop MR (1966) The role of algae in the nutrition of *Heteramoeba clara* Droop, with notes on *Oxyrrhis marina* Dujardin and *Philodina roseola* Ehrenberg. In: Barnes H (ed) some contemporary studies in marine science. George Allen and Unwin Ltd, London, pp 269–282

Droop MR, Pennock JF (1971) Terpenoid quinones and steroids in the nutrition of *Oxyrrhis marina*. J mar biol Ass UK 51: 455–470

Ederington MC, McManus GB, Harvey HR (1995) Trophic transfer of fatty acids, sterols and a triterpenoid alcohol between bacteria, a ciliate, and the copepod *Acartia tonsa*. Limnol Oceanogr 40: 860–867

Enright CT, Newkirk GF, Craigie JS, Castell JD (1986) Growth of juvenile *Ostrea edulis* L. fed *Chaetoceros gracilis* Schütt of varied chemical composition. J exp mar Biol Ecol 96: 15–26

Fenchel T (1987) Ecology of Protozoa: the biology of free-living phagotrophic protists. Brock/Springer, Madison/Berlin

Fingerman M (1987) The endocrine mechanism in crustaceans. J Crustacean Biol 7: 1–24

Fuller AKR (1990) The grazing and growth rates of some marine Protozoa measured in batch and continuous culture with particular reference to the heterotrophic dinoflagellate *Oxyrrhis marina*. PhD thesis, University of London, London

Gaines G, Elbrächter M (1987) Heterotrophic nutrition. In: Taylor FJR (ed) The biology of dinoflagellates. Botanical Monographs 21. Blackwell, Oxford, pp 244–268

Gibbs AG (1998) The role of lipid physical properties in lipid barriers. Am Zool 38: 268–279

Gifford DJ (1991) The protozoan–metazoan trophic link in pelagic ecosystems. J Protozool 38: 81–86

Gifford DJ, Dagg MJ (1991) The microzooplankton–mesozooplankton link: consumption of planktonic Protozoa by the calanoid copepods *Acartia tonsa* Dana and *Neocalanus plumchrus* Marukawa. Mar microb Fd Webs 5: 161–177

Goad LJ (1981) Sterol biosynthesis and metabolism in marine invertebrates. Pure appl Chem 51: 837–852

Grice K, Klein Breteler WCM, Schouten S, Grossi V, Leeuw JWD, Sinninghe Damsté JS (1998) The effects of zooplankton herbivory on biomarker proxy records. Paleoceanogr 13: 686–693

Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Chanley S (ed) Culture of marine invertebrate animals. Plenum Press, New York, pp 29–60

Hamamoto T, Takata N, Kudo T, Horikoshi K (1995) Characteristic presence of polyunsaturated fatty acids in marine psychrophilic vibrios. Fedn eur microbiol Soc (FEMS) Microbiol Lett 129: 51–56

Harrington GW, Beach DH, Dunham JE, Holz G (1970) The polyunsaturated fatty acids of marine dinoflagellates. J Protozool 17: 213–219

Harrington GW, Holz GGJ (1968) The monoenoic and docosahexaenoic fatty acids of a heterotrophic dinoflagellate. Biochim biophys Acta 164: 137–139

Harvey HR, Ederington MC, McManus GB (1997) Lipid composition of the marine ciliates *Pleuronema* sp. and *Fabrea salina*: shifts in response to changes in diet. J eukaryot Microbiol 44: 189–193

Heinbokel JF (1978) Studies on the functional role of tintinnids in the Southern California Bight. 1. Grazing and growth rates in laboratory cultures. Mar Biol 47: 177–189

Hirst AG, Lampitt RS (1998) Towards a global model of in situ weight-specific growth in marine planktonic copepods. Mar Biol 132: 247–257

Huntley M (1988) Feeding biology of *Calanus*: a new perspective. Hydrobiologia 167/168: 83–99

Jonasdottir SH, Fields D, Pantoja S (1995) Copepod egg production in Long Island Sound, USA, as a function of the chemical composition of seston. Mar Ecol Prog Ser 119: 87–98

Kiørboe T, Munk P, Richardson K, Christensen V, Paulsen H (1988) Plankton dynamics and larval herring growth, drift and survival in a frontal area. Mar Ecol Prog Ser 44: 205–219

Klein Breteler WCM, Gonzalez SR (1986) Culture and development of *Temora longicornis* (Copepoda, Calanoida) at different conditions of temperature and food. Syllogeus (Nat Mus Can) 58: 71–84

Klein Breteler WCM, Gonzalez SR (1988) Influence of temperature and food concentration on body size, weight and lipid content of two calanoid copepod species. Hydrobiologia 167/168: 201–210

Klein Breteler WCM, Laan M (1993) An apparatus for automatic counting and controlling density of pelagic food particles in cultures of marine organisms. Mar Biol 116: 169–174

Klein Breteler WCM, Schogt N (1994) Development of *Acartia clausi* (Copepoda, Calanoida) cultured at different conditions of temperature and food. Hydrobiologia 292/293: 469–479

Klein Breteler WCM, Schogt N, Gonzalez SR (1990) On the role of food quality in grazing and development of life stages, and genetic change of body size during cultivation of pelagic copepods. J exp mar Biol Ecol 135: 177–189

Klein Breteler WCM, Schogt N, van der Meer J (1994) The duration of copepod life stages estimated from stage-frequency data. J Plankton Res 16: 1039–1057

Kleppel GS (1992) Environmental regulation of feeding and egg production by *Acartia tonsa* off southern California. Mar Biol 112: 57–65

Kleppel GS (1993) On the diets of calanoid copepods. Mar Ecol Prog Ser 99: 183–195

Kleppel GS, Burkart CA (1995) Egg production and the nutritional environment of *Acartia tonsa*: the role of food quality in copepod nutrition. ICES J mar Sci 52: 297–304

Kleppel GS, Burkart CA, Houchin L (1998) Nutrition and the regulation of egg production in the calanoid copepod *Acartia tonsa*. Limnol Oceanogr 43: 1000–1007

Koski M, Klein Breteler W, Schogt N (1998) Effect of food quality on rate of growth and development of the pelagic copepod *Pseudocalanus elongatus* (Copepoda, Calanoida). Mar Ecol Prog Ser 170: 169–187

Langdon CJ, Waldock MJ (1981) The effect of algal and artificial diets on the growth and fatty composition of *Crassostrea gigas* spat. J mar biol Ass UK 61: 431–440

Lourenço SO, Barbarino E, Lanfer Marquez UM, Aidar E (1998) Distribution of intracellular nitrogen in marine microalgae: basis for the calculation of specific nitrogen-to-protein conversion factors. J Phycol 34: 798–811

Mayzaud P, Chanut JP, Ackman RG (1989) Seasonal changes of the biochemical composition of marine particulate matter with special reference to fatty acids and sterols. Mar Ecol Prog Ser 56: 189–204

Mayzaud P, Tirelli V, Bernard JM, Roche-Mayzaud O (1998) The influence of food quality on the nutritional acclimation of the copepod *Acartia clausi*. J mar Syst 15: 483–493

Müller-Navarra D (1995) Evidence that a highly unsaturated fatty acid limits *Daphnia* growth in nature. Arch Hydrobiol 132: 297–307

Nagata T, Takai K, Kawabata K, Nakanishi M, Urabe J (1996) The trophic transfer via a picoplankton–flagellate–copepod food chain during a picocyanobacterial bloom in Lake Biwa. Arch Hydrobiol 137: 145–160

Nichols DS, Hart P, Nichols PD, McMeekin TA (1996) Enrichment of the rotifer *Brachionus plicatilis* fed an Antarctic bacterium containing polyunsaturated fatty acids. Aquaculture, Amsterdam 147: 115–125

Norsker NH, Støttrup JG (1994) The importance of dietary HUFAs for fecundity and HUFA content in the harpacticoid, *Tisbe holothuriae* Humes. Aquaculture, Amsterdam 125: 155–166

Phillips NW (1984) Role of different microbes and substrates as potential suppliers of specific, essential nutrients to marine detritivores. Bull mar Sci 35: 283–298

Prahl FG, Eglinton G, Corner EDS, O'Hara SCM, Forsberg TEV (1984) Changes in plant lipids during passage through the gut of *Calanus*. J mar biol Ass UK 64: 317–334

Sargent JR, Parkes RJ, Mueller-Harvey J, Henderson RJ (1987) Lipid biomarkers in marine ecology. In: Sleigh MA (ed) Microbes in the sea. Ellis Horwood Ltd., Chichester, pp 119–138

Schouten S, Klein Breteler WCM, Blokker P, Schogt N, Rijpstra WIC, Grice K, Baas M, Sinninghe Damsté JS (1998) Biosynthetic effects on the stable carbon isotopic compositions of algal lipids: implications for deciphering the carbon isotopic biomarker record. Geochim cosmochim Acta 62: 1397–1406

Sherr EB, Sherr BF, Paffenhöfer GA (1986) Phagotrophic Protozoa as food for metazoans: a "missing" trophic link in marine pelagic food webs? Mar microb Fd Webs 1: 61–80

Sleigh M (1989) Protozoa and other protists. Edward Arnold, London

Stoecker DK, Mc Dowell Capuzzo J (1990) Predation on Protozoa: its importance to zooplankton. J Plankton Res 12: 891–908

Støttrup JG, Jensen J (1990) Influence of algal diet on feeding and egg-production of the calanoid copepod *Acartia tonsa* Dana. J exp mar Biol Ecol 141: 87–105

Sundbom M, Vrede T (1997) Effects of fatty acid and phosphorus content of food on the growth, survival and reproduction of *Daphnia*. Freshwat Biol 38: 665–674

Tong SM (1997) Heterotrophic flagellates and other protists from Southampton water, U.K. Ophelia 47: 71–131

Verity PG, Villareal TA (1986) The relative food value of diatoms, dinoflagellates, flagellates, and *Cyanobacteria* for tintinnid cilates. Arch Protistenk 131: 71–84

Walne PR (1970) Studies on the food value on nineteen genera of algae to juvenile bivalves of the genera *Ostrea*, *Crassostrea*, *Mercenaria*, and *Mytilus*. Fishery Invest (Lond) 26: 1–62

Weers PMM, Gulati RD (1997) Effect of the addition of polyunsaturated fatty acids to the diet on the growth and fecundity of *Daphnia galeata*. Freshwat Biol 38: 721–729

Yongmanitchai W, Ward OP (1989) Omega-3 fatty acids: alternative sources of production. Process Biochem 24: 117–125

Zhukova NV, Aizdaicher NA (1995) Fatty acid composition of 15 species of marine microalgae. Phytochem 39: 351–356