Mar Biol (2008) 155:23–36
DOI 10.1007/s00227-008-1003-2

ORIGINAL PAPER

Mechanism(s) of long chain n-3 essential fatty acid production in two species of heterotrophic protists: *Oxyrrhis marina* and *Gyrodinium dominans*

Eric D. Lund · Fu-Lin E. Chu · Ellen Harvey · Richard Adlof

Received: 31 January 2008 / Accepted: 10 May 2008 / Published online: 12 June 2008

Abstract As intermediaries, some heterotrophic protists can enhance the content of the long chain n-3 essential fatty acids (LCn-3EFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), of low food quality algae for subsequent use at higher trophic levels. However, the mechanisms that produce LCn-3EFAs are presently unknown, although LCn-3EFA production by heterotrophic protists at the phytoplankton–zooplankton interface may potentially affect the nutritional status of the pelagic system. We investigated whether the heterotrophic protists, *Oxyrrhis marina* and *Gyrodinium dominans*, produce LCn-3EFAs via elongation and desaturation of dietary LCn-3EFA precursors and/or synthesize LCn-3EFAs de novo by: (1) feeding the two heterotrophic protists with a prey deficient in n-3 fatty acids, (2) incubating them in medium containing ^{13}C-labeled sodium acetate, and (3) feeding the two protists gelatin acacia microspheres (GAMs) containing a deuterium-labeled LCn-3EFA precursor, linolenic acid [18:3(n-3)-d4]. Both *O. marina* and *G. dominans* synthesized EPA and DHA when fed the n-3 fatty acid-deficient prey, *Perkinsus marinus*, a parasitic protozoan. *O. marina*, but not *G. dominans* utilized ^{13}C-labeled acetate from the medium to produce uniformly labeled fatty acids, including DHA. Both heterotroph species consumed GAMs containing 18:3(n-3)-d4 and catabolized 18:3(n-3)-d4 to 16:3(n-3)-d4 and 14:3(n-3)-d4, while no 20 or 22 carbon metabolites of 18:3(n-3)-d4 were detected. These results suggest that *O. marina* and *G. dominans* do not elongate and desaturate dietary LCn-3EFA precursors to produce LCn-3EFAs, but rather they produce LCn-3EFAs de novo, possibly via a polyketide synthesis pathway.

Communicated by U.-G. Berninger.

E. D. Lund · F.-L. E. Chu (✉) · E. Harvey
Virginia Institute of Marine Science,
College of William and Mary, P. O. 1346,
Gloucester Point, VA 23062, USA
e-mail: chu@vims.edu

R. Adlof
USDA, ARS, NCAUR, 1815 N. University,
Peoria, IL 61604, USA

Introduction

Protists, such as ciliates and heterotrophic and mixotrophic dinoflagellates, are important food sources for zooplankton in the pelagic marine environment (Dolan 1991; Fessenden and Cowles 1994; Zeldis et al. 2002). Heterotrophic protists utilize a wide variety of food sources including micro-, nano-, pico-algae, bacteria, detritus, and dissolved organic matter (DOM). As intermediates, they provide a critical link in the flow of carbon and nutrients between the microbial loop and higher trophic levels.

One particularly crucial class of nutrients produced at the lowest trophic levels is long chain essential fatty acids, which are critical structural components and precursors to signaling molecules and involved in many diverse biological and biochemical processes. These long chain essential fatty acids include the long chain n-3 essential fatty acids (LCn-3EFAs), docosahexaenoic acid [DHA, 22:6(n-3)] and eicosapentaenoic acid [EPA, 20:5(n-3)] as well as the long chain n-6 essential fatty acid, arachidonic acid [AA, 20(n-6)]. These fatty acids cannot be synthesized in sufficient quantities by higher animals and must be acquired from dietary sources. Due to the physiological importance of

LCn-3EFAs in marine organisms and the species-specific variability of their abundance in marine algae they are potentially limiting nutrients of crustaceans and fish in pelagic ecosystems and may be important factors in determining the efficiency of carbon transfer at the phytoplankton–heterotrophic protist interface and reproduction and recruitment of organisms at higher trophic levels.

In addition to being an important carbon source, as intermediates, some heterotrophic protists do not only enhance the carbon transfer efficiency, but improve poor quality algal food for subsequent use at higher trophic levels by enhancing the biochemical constituents of the algal prey. The latter phenomenon is called "trophic upgrading" (Klein-Breteler et al. 1999; Bec et al. 2003, 2006; Tang and Taal 2005; Broglio et al. 2003; Veloza et al. 2006). "Trophic upgrading" by heterotrophic protists has been noted in both marine (e.g., Klein-Breteler et al. 1999; Tang and Taal 2005; Broglio et al. 2003; Veloza et al. 2006) and fresh water (Bec et al. 2003, 2006; Boechat and Adrian 2005; Martin-Creuzburg et al. 2005) systems.

Recent studies of feeding, growth and reproduction of calanoid copepods suggest that some heterotrophic protists are a preferred prey relative to algae (Dolan 1991; Fessenden and Cowles 1994; Zeldis et al. 2002). Copepods feeding on heterotrophic protists can exhibit higher growth and reproduction than copepods fed low food quality algae alone (Klein-Breteler et al. 1999; Tang et al. 2001; Broglio et al. 2003). Calanoid copepods fed a diet consisting of only the LCn-3EFA-deficient alga *Dunaliella tertiolecta* failed to reproduce and died within several days (Tang et al. 2001). However, when the heterotrophic dinoflagellate protists *Oxyrrhis marina* or *Gyrodinium dominans* were raised on a diet of *D. tertiolecta* and either of the protists were then fed to calanoid copepods they supported copepod growth and reproduction (Tang et al. 2001). Lipid analysis revealed that the essential fatty acids, EPA and DHA, which were not present in the algal prey *D. tertiolecta* were present in the predators, *O. marina* and *G. dominans*. Also, when *O. marina* and *G. dominans* were fed the DHA-containing alga *Rhodomonas salina* they had elevated levels of DHA relative to their algal prey (Klein-Breteler et al. 1999; Tang and Taal 2005; Veloza et al. 2006). Similarly, "trophic upgrading" of food quality by freshwater heterotrophic flagellates for the metazooplankton, *Daphnia hyalina* and *Daphnia magna* (Bec et al. 2003, 2006) have also been reported. Increased quantity and diversity of n-3 series PUFA were noted in the heterotrophic flagellate *Aulacomonas submarina* fed microalgae containing LCn-3EFAs and/or LCn-3EFA precursors (Bec et al. 2003). Production of sterols and LCn-3EFAs (EPA and DHA) and LCn-6EFA (AA) by the heterotrophic nanoflagellate, *Paraphysomonas* sp fed various algal diets has been implicated as being important in trophic upgrading (Bec et al. 2006). The presence of *Paraphysomonas* sp. as an intermediate increased both somatic growth and reproduction of *D. magna*.

All of the above studies suggest that some heterotrophic protists as intermediate prey enhance the nutritional value of low food quality algae, by "upgrading" the content of LCn-3EFAs and sterols, for subsequent use by higher trophic levels. In the marine environment trophic upgrading by heterotrophic protists has been most strongly linked to the increased LCn-3EFAs (Klein-Breteler et al.1999; Tang et al. 2001; Broglio et al. 2003; Veloza et al. 2006). Due to their role as trophic intermediates between autotrophs and mesozooplankton, heterotrophic protists may at times be important producers of LCn-3EFA in marine pelagic ecosystems.

Previously, we have shown that the two marine heterotrophic protists, *O. marina* and *G. dominans*, efficiently produce EPA and DHA when fed the LCn-3EFA-deficient prey *D. tertiolecta* (Chu et al. 2008). Furthermore, production of LCn-3EFAs by heterotrophic protists may contribute significant quantities of LCn-3EFAs to the pool of available zooplankton prey items and also alter the DHA:EPA ratio relative to exclusively autotrophic prey items (Chu et al. 2008). The mechanisms and chemical processes of LCn-3EFA "upgrading" in *O. marina* and *G. dominans*, however, are presently not known.

The prevailing view on the mechanisms whereby marine heterotrophic protists produce LCn-3EFAs when consuming prey containing LCn-3EFAs or deficient of LCn-3EFAs have been that they selectively sequester dietary LCn-3EFAs, and/or elongate and desaturate 16 and 18 carbon (n-3) fatty acids from their diets to produce EPA and DHA (Klein-Breteler et al. 1999; Broglio et al. 2003; Veloza et al. 2006). A third possible mechanism that has generally been overlooked is that protists produce LCn-3EFAs by synthesizing them de novo.

The objective of this study was to determine mechanism(s) and potential substrates utilized by the two microzooplankton, *O. marina* and *G. dominans*, to produce LCn-3EFAs through the use of n-3 fatty acid deficient prey and stable isotope-labeled substrates. Understanding the pathways and substrates used by heterotrophic protists for the synthesis of LCn-3EFAs may help define what carbon sources can be converted into these essential nutrients. This information may have important ramifications for better understanding the factors regulating the production of zooplankton in the marine environment.

Materials and methods

Growth and fatty acid profiles of *O. marina* and *G. dominans* fed a n-3 fatty acid-deficient prey

Culture and preparation of n-3 fatty acid-deficient prey for heterotroph feeding experiments

In order to test whether *O. marina* or *G. dominans* requires dietary n-3 fatty acids to synthesize EPA and DHA, feeding experiments were conducted using *Perkinsus marinus*, a protozoan parasite of the eastern oyster, *Crassostrea virginica*, as a prey species. *P. marinus* is able to synthesize a range of saturated and unsaturated fatty acids including the essential fatty acid, AA [20:4(n-6)], but not n-3 fatty acids (Soudant and Chu 2001; Lund et al. 2007). *P. marinus* cells (trophozoite/meront stage) were cultured at 28°C in 1 L culture flasks in a defined, detergent-free medium containing cholesterol (5 μg ml^{-1}) as the only lipid component (Lund et al. 2007). Prior to use in heterotroph feeding experiments, *P. marinus* cultures were centrifuged at 2,000×*g* for 15 min, the supernatant was removed and the cell pellet was resuspended in fresh f/2 medium. Cell densities of *P. marinus* cells resuspended in f/2 medium were determined by four replicate counts in a hemocytometer.

Fatty acid profiles of O. marina Dujardin and G. dominans Hulbert fed n-3 fatty acid-deficient prey

Cell densities of prey-depleted *O. marina* cultures that had been previously maintained on the algae *D. tertiolecta* (CCMP 1320) were determined by direct count of culture aliquots fixed with Lugols solution using a 1.0 ml Sedgewick-Rafter chamber. Prey-depleted *O. marina* cells were added to sixteen 300 ml glass culture bottles containing fresh f/2 medium at a density of 500 cells ml^{-1} along with *P. marinus* cells at a density of 300,000 cells ml^{-1}. Bottles containing *O. marina* and *P. marinus* were maintained in the dark at 19°C on a rotating plankton wheel at 1.5 rpm until harvested. The inoculated mixed cultures (*O. marina* + *P. marinus*) ($n = 4$ bottles) were collected on acetone washed GF/F filters at the time of inoculation, and 1, 2, and 3 days after inoculation ($n = 4$ bottles for each sampling date) for fatty acid analysis. Additionally, at the start of the feeding experiment, four replicate samples of prey-depleted *O. marina* (3.0×10^5 cells per replicate) and *P. marinus* cells (9×10^7 per replicate) were also collected on acetone washed GF/F filters for subsequent lipid extraction and fatty acid analysis.

Another feeding experiment using *P. marinus* as a prey species was conducted with *G. dominans* as the heterotrophic predator. Experimental design, inoculation densities of predator and prey species and sampling protocol were identical to the *O. marina–P. marinus* feeding experiment described above.

Determination of fatty acid composition and content of samples

Total lipids were extracted from samples according to the procedure of Bligh and Dyer (1959). Extracted lipids were transesterified in 8-ml Wheaton vials containing 20 μg internal standard (23:0), with 10% BF_3 (w/w) in methanol for 15 min at 95–100°C (Metcalfe and Schmitz 1961). The fatty acid methyl esters (FAMEs) were extracted with carbon disulfide (Marty et al. 1992) which was evaporated under a stream of nitrogen. Samples were then redissolved in hexane for analysis.

Fatty acid methyl esters derived from samples were analyzed for fatty acid profiles and contents using GC/FID (Varian 3800, equipped with a flame ionization detector; Varian Analytical Instruments, Sunnydale, CA) using a DB-WAX capillary column (25 m × 0.32 mm; 0.2 μm film thickness; J&W Scientific, Folsom, CA). Identification of FAMEs was based on the comparison of their retention times with those of known standards and confirmed by gas chromatograph-mass spectrometry (GC/MS).

Incorporation of sodium ^{13}C acetate in f/2 medium into fatty acids of *O. marina* and *G. dominans*

Incubation of prey-depleted protists with ^{13}C-labeled acetate

To determine if *O. marina* and *G. dominans* are capable of using the two carbon substrate, ^{13}C-labeled acetate, provided in the medium for fatty acid synthesis, we tested the "optimal" concentrations of ^{13}C-acetate to be used by the protists. Prey-depleted protist cultures (3 days since last feeding) of each species that had been previously maintained on *D. tertiolecta* were incubated in media containing the substrates sodium 1,2 ^{13}C acetate (Cambridge Isotope Laboratories, Andover MA) or- non-labeled sodium acetate. Sodium acetate substrates (labeled or unlabeled) were added at concentrations of 6 mg ml^{-1}, 200 μg ml^{-1}, and 25 μg ml^{-1} to 300 ml culture bottles containing 3,450 *G. dominans* ml^{-1} ($n = 3$) or 2,000 *O. marina* ml^{-1} ($n = 3$). Culture bottles were maintained in the dark at 19°C on a plankton wheel (1.5 rpm) for 24 h until harvested. At the time of harvest, 4 ml from each bottle was used to determine cell viability and culture density. The remainder was collected on acetone-washed GF/F filters for subsequent lipid extraction and FAME derivatization (described above) to determine incorporation of ^{13}C-labeled acetate in fatty acids by GC/FID and GC/MS analyses.

Determination of incorporation of ^{13}C-labeled acetate in FAMEs

To detect incorporation of ^{13}C-labeled acetate, FAMEs were analyzed qualitatively and quantitatively by gas chromatography/mass spectrometry (GC/MS) with a Varian 3400 gas chromatograph equipped with a Varian Saturn 4D GC/MS/MS ion trap detector. Methane was used as the reagent gas for positive chemical ionization (PCI). The same column used for GC/FID analysis of the FAME samples (J&W DB-WAX, 25 m × 0.32 mm; 0.2 μm film thickness) was used for GC/MS analysis. Carrier gas (helium) flow rate was 1 ml min^{-1}. Injection port temperature was 230°C and the interface was 250°C. The column was temperature programmed from an initial temperature of 60°C for a 4 min hold, followed by a 30°C min^{-1} increase to 150 and 2°C min^{-1} to 220°C. Data was collected and processed using Varian Saturn GC/MS software version 5.2. FAMEs were identified by retention time relative to known standards, fragmentation pattern and mass of the molecular ion. FAMEs containing ^{13}C derived from acetate were quantified using standard curves constructed for each FAME standard and ratio with internal standard (23:0). The standard curves were created using four concentrations of each FAME comparable to the concentration of the sample, along with a fixed amount (20 μg) of the C23:0 internal standard. The molecular ions in spectra of each FAME were used to quantify masses containing exogenous ^{13}C relative to the native molecule. Samples from cultures containing non-labeled sodium acetate were also analyzed in an identical manner as negative controls for ^{13}C incorporation.

MS spectra of highly unsaturated fatty acids have more extensive fragmentation than saturated fatty acids. This results in little or no production of M^+ ions (molecular ion) which are necessary for determining the amount of ^{13}C incorporation in the fatty acids. To facilitate identification of highly unsaturated FAMEs containing ^{13}C from the ^{13}C acetate substrate FAME samples were hydrogenated to saturated fatty acid methyl ester congeners and reanalyzed by GC/MS. FAME samples in hexane were dried under a stream of nitrogen and resuspended in a pyrex test tube containing 3 ml of methanol and 2 mg of platinum oxide (Adams Reagent). Samples were then bubbled with hydrogen gas for 2 h to hydrogenate them (Christie 1989). Hydrogenated FAMEs were dried under a stream of nitrogen and resuspended in hexane for GC/FID and GC/MS analysis to quantify the incorporation of any ^{13}C from labeled acetate.

Incorporation and metabolism of 18:3(n-3)-d4 contained in gelatin–acacia microspheres by *O. marina* and *G. dominans*

Results from a preliminary experiment revealed that both *O. marina* and *G. dominans* were unable to use EPA and DHA precursors, such as the deuterium-labeled18:3(n-3)-d4 and 20:3(n-3)-d8, which were previously synthesized at USDA by the co-author Richard Adlof and his associates, incorporated in the culture medium. Therefore, we used microcapsules to mimic microalgae to deliver labeled substrates to the protists through ingestion. We encapsulated [18:3(n-3)6,6,7,7,-d4] (18:3(n-3)-d4) in gelatin–acacia capsules and fed the capsules together with the EPA and DHA deficient alga, *D. tertiolecta*, to *G. dominans* or *O. marina* to determine the uptake and metabolic fate of this fatty acid. The sizes of gelatin acacia microspheres (GAMs) ranged from 5 to 20 microns, which are of similar particle size to heterotrophic protists' prey items in nature.

Preparation of GAMs containing deuterium-labeled and non-labeled 18:3(n-3)

Gelatin acacia microspheres (GAMs) were prepared according to the method described by Chu et al. (1987). Briefly, a 2% (w/v) solution of gelatin and a 2% (w/v) solution of acacia were heated to 40°C. Ten ml of each solution were combined and stirred at approximately 500 rpm. A lipid mixture containing either 450 mg olive oil and 50 mg non-labeled 18:3(n-3) or 450 mg olive oil and 50 mg 18:3(n-3)-d4 was then added to the gelatin–acacia solution. The gelatin–acacia–lipid mixture was next homogenized with an Ultra-Turax T-ZS tissue homogenizer for 2 min. The homogenized mixture was then stirred at 500 rpm and the pH was reduced to 3.9 by the drop-wise addition of 0.2 N HCl. Stirring was then reduced to approximately 100 rpm at 40°C for 1 h. The pH of the solution was then raised to 9.3 by the slow addition of 0.5 M NaOH and stirred for an additional 5 min. The solution containing the GAMs was then added to 80 ml of ice-cold DI water and kept on ice for 2 h. The GAMs were then centrifuged at 2,000 × *g* for 4 min and the water under the buoyant GAMs was removed and the GAMs were washed with 80 ml ice-cold DI water and centrifuged again. All but the last 20 ml of the water under the GAMs was removed. The GAMs were then vortexed and stored at 4°C until used for feeding studies. Three 0.5 ml aliquots from the 18:3(n-3) GAMs and 18:3(n-3)-d4 GAMs were removed for lipid extraction and subsequent fatty acid analysis. Additional aliquots of 100 μl GAMs were removed for microscopic determination of GAM particle size range.

Prior to feeding the protists with GAMs, the ingestion of GAMs by *O. marina* and *G. dominans* was tested by feeding them "Sudan red stained" GAMs and confirmed by examination under a light microscope. "Sudan red stained" GAMs were prepared using lipid containing a red fat soluble dye, Sudan red. One gram of olive oil was mixed with 0.5 g Sudan IV and heated for 5 min at 35°C. The oil–dye mixture was then centrifuged at 2,000 × *g* for 20 min and

500 mg of the supernatant was used as the lipid component of the GAMs. GAMs containing the dyed olive oil were prepared as described above.

Ingestion/consumption and metabolism of GAM containing [18:3(n-3)]-d4 by O. marina and G. dominans

In the GAM feeding experiments, prey-depleted *O. marina* was inoculated into 300 ml replicate bottles at a concentration of 500 cells ml^{-1} (1.5×10^5 per bottle) along with 50,000 *D. tertiolecta* ml^{-1} (1.5×10^7 per bottle). There were two treatment groups ($n = 4$ replicate bottles per treatment): one fed with GAMs containing unlabeled 18:3n-3 (control) and the other fed with GAMs containing 18:3(n-3)-d4. In the control group, each bottle received 0.5 ml unlabeled 18:3(n-3) GAMs and in the treatment group, each bottle received 0.5 ml 18:3(n-3)-d4 GAMs. All culture bottles were then maintained at 19°C in the dark on a plankton wheel at 1.5 rpm overnight. Each culture bottle received additional 0.5 ml GAM containing labeled or unlabeled 18:3(n-3) at 16, 19, 24, and 40 h. All cultures were harvested at 45 h after the start of the experiment. Prior to harvest, triplicate aliquots of 1.0 ml from each replicate bottle were taken to determine protist densities before letting the bottles sit upright on a benchtop for 45 min to allow unconsumed GAMs to float toward the surface. Next, the top 25 ml of culture medium was removed from each bottle and the remainder was filtered onto acetone-washed GF/F filters for subsequent lipid extraction, FAME derivitization and GC/FID and GC/MS analyses. An experiment was also carried out using replicate cultures of *G. dominans* fed with *D. tertiolecta* and GAMs. This experiment was identical to the above-described *O. marina* -GAM experiment except that at each GAM feeding time 0.25 ml of GAM were added to *G. dominans* cultures instead of the 0.5 ml of GAMs added to *O. marina* cultures.

To identify metabolites of 18:3(n-3)-d4, FAMEs derived from GAMs containing deuterium labeled or unlabeled 18:3(n-3) and from the control and treatment groups were subjected to argenation TLC analysis (Kates 1972) to separate polyunsaturated fatty acids from monounsaturated fatty acids and saturated fatty acids. This was done for two reasons: (1) 90% of the GAM lipid was olive oil which itself contains approximately 90% 18:1(n-9). The TLC step gets rid of this component and allows for concentrating the PUFAs for GC-FID and GC-MS analysis; and (2) This step allows for further confirmation that the peaks identified as 18:3(n-3) metabolites were identified correctly, since only PUFAs remained in the TLC fraction saved for analysis of 18:3(n-3) metabolites. Briefly, a 20 cm silica gel TLC plate containing 5% silver nitrate (Alltech#28011) was activated by heating at 110°C for 30 min. Two pooled replicates from each treatment were dried under a stream of nitrogen, resuspended in 30 µl hexane and spotted on the activated TLC plate. The plate was developed in petroleum ether:ethyl ether (95:5) for 1 h. Fractions containing polyunsaturated, monounsaturated, and saturated FAMEs were visualized by dichloroflourescein and scraped from the plate into round bottomed 10 ml test tubes and eluted with 3×1.5 ml ethyl ether. Samples were then dried under nitrogen, resuspended in 1 ml hexane and reanalyzed by GC/FID and GC/MS. Fractions containing polyunsaturated FAMEs were then hydrogenated as described previously. Hydrogenated FAMEs were reanalyzed by GC/FID and GC/MS to determine the existence of any 14, 16, 20, or 22 carbon fatty acid metabolites of the 18:3(n-3)-d4 substrate. Such metabolites contain four deuteriums and have a molecular weight 4 Da higher than non-deuterated FAMEs originating from other sources.

Statistical analysis

Significant differences of the culture content of individual fatty acids, n-3 fatty acids, n-6 fatty acids and total fatty acids of *O. marina* and *G. dominans* cultures fed *P. marinus* were determined by analysis of variance (1-way ANOVA). When results were significant ($P < 0.05$), Tukeys test was employed to determine significant differences between each sampling point.

Results

Fatty acid profiles and content of *O. marina* and *G. dominans* fed *P. marinus*

Fatty acid composition of O. marina and G. dominans inoculum cultures

The prey-depleted *Oxyrrhis marinus* culture used for inoculation was characterized by a high weight percentage of saturated fatty acids and lesser amounts of monounsaturated and polyunsaturated fatty acids. The saturated fatty acids 14:0, 16:0, and 18:0 and monounsaturates accounted for approximately 60 and 10% of total fatty acids, respectively (Table 1). The polyunsaturated fatty acids were generally characterized by a very high n-3:n-6 ratio of approximately 10. The most abundant polyunsaturates were 18:3(n-3) and 22:6(n-3) (12 and 11%, respectively) with lesser amounts (<3%) of 18:2(n-6) and 20:2(n-9) comprising the rest of the polyunsaturates.

The prey-depleted *G. dominans* culture used for inoculation was similar to that of the *O. marinus* culture in overall fatty acid composition. Saturated fatty acids accounted for more than 80% of total fatty acids (Table 1). The monounsaturates 18:1(n-9) and 18:1(n-7) together comprised less

Table 1 Percent fatty acid compositions of prey-depleted inoculums of the heterotrophic protists *Oxyrrhis marina* and *Gyrodimium dominans* and their n-3 fatty acid deficient prey, *Perkinsus marinus* ($n = 4$)

FAME (%)	*Oxyrrhis marina*	*Gyrodinium dominans*	*Perkinsus marinus*
14:0	2.6 ± 0.4	2.9 ± 0.5	4.5 ± 0.2
16:0	28.3 ± 1.0	32.2 ± 2.4	13.8 ± 1.3
18:0	29.4 ± 8.3	46.7 ± 4.6	11.2 ± 1.7
20:0	ND	ND	3.0 ± 0.2
22:0	ND	ND	2.7 ± 0.1
24:0	ND	ND	7.9 ± 0.6
16:1(n-9)	ND	ND	0.4 ± 0.4
16:1(n-7)	ND	ND	1.6 ± 0.1
18:1(n-9)	5.8 ± 1.4	6.1 ± 3.2	16.2 ± 2.7
18:1(n-7)	4.0 ± 1.1	1.7 ± 0.3	0.7 ± 0.5
20:1(n-11)	ND	ND	1.2 ± 0.1
20:1(n-9)	ND	ND	3.9 ± 0.7
20:1(n-7)	ND	ND	2.4 ± 2.9
22:1(n-11 + 9)	ND	ND	1.2 ± 0.3
20:2(n-9)	0.5 ± 0.9	ND	2.2 ± 1.4
18:2(n-6)	2.3 ± 0.6	ND	4.4 ± 0.8
20:2(n-6)	ND	ND	1.4 ± 0.4
20:3(n-6)	ND	ND	0.7 ± 0.6
20:4(n-6)	ND	ND	16.4 ± 2.6
18:3(n-3)	12.4 ± 2.8	1.1 ± 0.7	ND
20:5(n-3)	ND	ND	ND
22:6(n-3)	11.5 ± 2.9	2.2 ± 0.4	ND
Total n-6	2.3 ± 0.6	ND	23.0 ± 3.5
Total n-3	23.9 ± 5.6	3.3 ± 1.1	ND

ND ≤0.1%

than 8% of total fatty acids while the two detectable polyunsaturated fatty acids, 22:6(n-3) and 18:3(n-3) comprised 2 and 1% of total fatty acids. No n-6 fatty acids were detected in the *G. dominans* inoculum.

Perkinsus marinus cells used as prey for the two heterotroph species were characterized by high levels of n-6 polyunsaturated fatty acids (23% of total fatty acids) and no detectable n-3 fatty acids (Table 1). The four most abundant fatty acids in *P. marinus* were 20:4(n-6) (16%), 18:1(n-9) (16%), 16:0 (14%), and 18:0 (11%).

Growth and fatty acid profile and content of O. marina cultures fed P. marinus

Oxyrrhis marina cultures readily consumed *P. marinus* cells and increased more than tenfold over the course of the experiment, from an initial inoculation concentration of 500 cells ml^{-1} up to 5,210 ± 159 cells ml^{-1} on day 3 (Table 2). *P. marinus* cell density decreased from an initial inoculation density of 300,000 cells ml^{-1} to below 40,000 cells ml^{-1} by day 3.

Total fatty acids in the cultures decreased over the course of the experiment from an initial value of 159.2 ± 30.4 μg at inoculation to 110.5 ± 22.6 μg at day 3 (Table 2). All of the 18–24 carbon saturated and 20–22 carbon monounsaturated fatty acids, except 20:1(n-7) decreased significantly by day 2 and decreased further by day 3.

Polyunsaturated fatty acids in the *O. marina–P. marinus* cultures exhibited significant changes in n-3 and n-6 composition over the course of the experiment. The initial *O. marina–P. marinus* cultures at inoculation contained nearly ten times as much n-6 fatty acids as n-3 fatty acids (38.1 ± 11.9 and 4.3 ± 1.7 μg, respectively) (Table 2). By day 3 the total n-6 fatty acid content had dropped significantly to 20.8 ± 4.4 μg, while the n-3 fatty acid content had increased nearly fivefold to 19.3 ± 3.2 μg.

The small amount of 18:3(n-3) present in the inoculum (1.6 ± 1.2 μg) was no longer detectable by day 2. The only other n-3 fatty acids present were 20:5(n-3) and 22:6(n-3). EPA [20:5(n-3)] was not detectable in the inoculums, but increased to 2.3 ± 0.5 μg by day 2. DHA [22:6(n-3)] increased significantly from an initial inoculation concentration of 2.7 ± 0.5–17.4 ± 3.0 μg on day 2. The DHA:EPA ratio was very high throughout the experiment and increased progressively on each sampling day (5.8, 7.5, and 9.2 for days 1, 2, and 3, respectively).

Growth and fatty acid profile and content of G. dominans cultures fed P. marinus

Gyrodinium dominans grazed down the *P. marinus* prey and its density increased over sevenfold after 3 days, from an initial concentration of 500 cells ml^{-1} up to 3,750 ± 181 cells ml^{-1} on day 3 (Table 3). *P. marinus* cell density decreased from 300,000 cells ml^{-1} to below 50,000 cells ml^{-1} by day 3.

The content of total fatty acids did not change significantly over the 3 day experiment, but the composition of the fatty acids did change (Table 3). The saturated fatty acids 20:0, 22:0, and 24:0 and the monounsaturated fatty acids 20:1(n-9), and 22:1 decreased significantly by day 1 and the decrease continued to day 3, while the monounsaturates 16:1(n-9) and 18:1(n-7) both increased from the time of inoculation to day 3 (Table 3).

The level of n-6 fatty acids present in the cultures did not change significantly over the course of the study, but total n-3 fatty acids increased with each sampling, from an initial level of 0.8 ± 0.1 to 21.0 ± 7.4 μg on day 3 (Table 3). The increase in total n-3 fatty acid content was attributable to the production of EPA [20:5(n-3)] and DHA [22:6(n-3)]. EPA and DHA content of the cultures increased from <0.5 μg and 0.8 ± 0.1 μg, respectively, at inoculation to 4.5 ± 1.1 μg and 16.4 ± 8.2 μg at day 3 (Table 3).

Table 2 Micrograms of fatty acids (mean ± SD) in *Oxyrrhis marina* cultures fed *Perkinsus marinus*. Values are for 300 ml cultures inoculated with 500 *O. marina* ml^{-1} and 300,000 *P. marinus* ml^{-1} ($n = 4$)

FAME (μg)	Initial Om-Pm	Day 1 Om-Pm	Day 2 Om-Pm	Day 3 Om-Pm
14:0	5.1 ± 1.2ab	5.4 ± 0.7a	4.5 ± 0.8ab	3.3 ± 0.7b
16:0	23.7 ± 5.4	25.9 ± 2.2	23.7 ± 2.9	21.1 ± 3.2
18:0	15.7 ± 3.4a	18.7 ± 2.3a	8.1 ± 1.0b	7.4 ± 1.4b
20:0	4.0 ± 0.6a	3.0 ± 0.2b	1.7 ± 0.3c	1.5 ± 0.2c
22:0	3.7 ± 0.6a	2.7 ± 0.3b	1.7 ± 0.3c	1.6 ± 0.3c
24:0	12.6 ± 2.1a	9.1 ± 1.0b	6.3 ± 0.8c	5.8 ± 0.8c
16:1(n-9)	1.4 ± 0.6	0.7 ± 0.1	1.7 ± 0.9	1.3 ± 0.1
16:1(n-7)	1.0 ± 0.4b	2.0 ± 0.3a	1.5 ± 0.3ab	1.0 ± 0.4b
18:1(n-9)	19.0 ± 6.2	17.8 ± 1.3	14.7 ± 1.7	11.6 ± 2.9
18:1(n-7)	2.4 ± 0.6b	7.0 ± 0.9a	6.4 ± 1.6a	4.7 ± 1.6ab
20:1(n-11)	2.4 ± 0.5a	1.8 ± 0.1b	1.2 ± 0.2c	1.1 ± 0.2c
20:1(n-9)	3.7 ± 0.7a	2.7 ± 0.3b	1.7 ± 0.2c	1.5 ± 0.3c
20:1(n-7)	2.2 ± 2.0	0.9 ± 0.1	0.6 ± 0.4	0.3 ± 0.4
22:1(n-11 + 9)	2.2 ± 0.8a	1.7 ± 0.3b	0.7 ± 0.8c	0.7 ± 0.8c
20:2(n-9)	2.1 ± 0.3a	1.6 ± 0.2b	1.4 ± 0.3c	1.2 ± 0.3c
18:2(n-6)	3.7 ± 1.2	3.4 ± 0.3	3.7 ± 1.2	2.6 ± 1.1
20:2(n-6)	1.3 ± 0.5	1.2 ± 0.1	1.5 ± 1.1	0.8 ± 0.6
20:3(n-6)	2.0 ± 0.6a	1.7 ± 0.2ab	1.1 ± 0.1b	1.0 ± 0.2b
20:4(n-6)	31.1 ± 9.7a	26.1 ± 3.2ab	17.9 ± 2.6b	16.3 ± 3.1b
18:3(n-3)	1.6 ± 1.2	1.6 ± 0.2	ND	ND
20:5(n-3)	ND	1.2 ± 0.1b	2.3 ± 0.5a	1.9 ± 0.2a
22:6(n-3)	2.7 ± 0.5b	7.0 ± 0.4b	17.3 ± 6.2a	17.4 ± 3.0a
Total n-6	38.1 ± 11.9a	32.4 ± 3.8ab	24.2 ± 4.7ab	20.8 ± 4.4b
Total n-3	4.3 ± 1.7b	9.8 ± 0.5b	19.6 ± 5.8a	19.3 ± 3.2a
Total	159.2 ± 30.4a	151.8 ± 14.4ab	128.4 ± 21.2ab	110.5 ± 22.6b
Om ml^{-1}	500	1,377 ± 27	4,701 ± 448	5,210 ± 159
Pm/ml^{-1}	300,000	49,995 ± 2,619	43,725 ± 4,268	39,222 ± 9,555

Letters denote significant differences at the $P < 0.05$ level

Om Oxyrrhis marina, *Pm Perkinsus marinus*, *ND* ≤0.2 μg

DHA:EPA ratios of the cultures were 3.2, 2.5, and 3.6 for days 1–3, respectively.

Total heterotroph carbon content increased nearly tenfold from inoculation to day 3 in both *O. marina* and *G. dominans* cultures, roughly parallel to the increase in DHA content (6.4 and 20.5-fold increases in *O. marina* and *G. dominans*, respectively) (Table 4). While EPA content of cultures also greatly increased, very low levels of EPA in heterotroph inoculums (<0.2 μg) preclude direct comparisons to changes in heterotroph biomass (Table 4). The increased heterotroph biomass, increased LCn-3EFA content and decreased cell density of n-3 fatty acid-deficient prey at day 3 relative to the inoculums are consistent with the heterotrophs producing LCn-3EFA from carbon originating from n-3 fatty acid-deficient prey. These results suggest that dietary LCn-3EFA precursors, such as 18:3n-3 and 16:4n-3, are not required for LCn-3EFA production by *O. marina* and *G. dominans*.

Utilization of ^{13}C acetate from medium by *O. marina* and *G. dominans*

Through several incubation trials, it was found that: (1) a concentration of 6 mg ml^{-1} ^{13}C acetate was lethal to both protist species, (2) the concentration of 25 μg ml^{-1} ^{13}C acetate supported limited protist growth and no incorporation of ^{13}C in fatty acids was noted through GC/MS analysis and (3) the concentration of 200 μg ml^{-1} ^{13}C acetate caused *G. dominans* cultures to crash within 24 h, but resulted in proliferation of *O. marina* and significant incorporation of ^{13}C label into at least five different fatty acids: 14:0, 16:0, 16:1(n-7), 18:0, and 22:6(n-3) (Table 5). The most abundant ^{13}C-containing fatty acid was 14:0, followed by 16:0, 18:1(n-7), 16:1(n-7) and 22:6(n-3), respectively (Table 5). DHA was the only 22 carbon fatty acid containing ^{13}C. It is highly notable and unusual that all fatty acid molecules contained either all ^{13}C (uniformly labeled) or all ^{12}C (native) (Fig. 1). No quantities

Table 3 Micrograms of fatty acids (mean ± SD) in *Gyrodinium dominans* cultures fed *Perkinsus marinus*. Values are for 300 ml cultures inoculated with 500 *G. dominans* ml^{-1} and 300,000 *P. marinus* ml^{-1} ($n = 4$)

FAME (μg)	Initial Gd–Pm	Day 1 Gd–Pm	Day 2 Gd–Pm	Day 3 Gd–Pm
14:0	5.1 ± 1.1	4.9 ± 1.4	8.0 ± 3.0	7.9 ± 2.0
16:0	24.4 ± 4.2	19.7 ± 4.6	33.0 ± 12.4	29.6 ± 3.3
18:0	20.9 ± 3.0a	9.5 ± 1.9b	25.1 ± 9.5a	9.6 ± 0.7b
20:0	3.7 ± 0.7a	2.2 ± 0.4b	1.9 ± 0.4b	1.7 ± 0.3b
22:0	3.2 ± 0.5a	2.0 ± 0.3b	1.5 ± 0.1b	1.2 ± 0.8b
24:0	11.2 ± 2.0a	7.3 ± 1.1b	5.1 ± 0.1b	5.3 ± 1.2b
16:1(n-9)	0.7 ± 0.5b	1.6 ± 0.7ab	0.8 ± 0.6b	2.6 ± 0.8a
16:1(n-7)	1.1 ± 0.2	1.7 ± 0.6	2.2 ± 0.7	1.7 ± 0.3
18:1(n-9)	18.4 ± 4.0	14.1 ± 4.9	17.3 ± 6.7	18.3 ± 1.4
18:1(n-7)	1.5 ± 0.3b	3.7 ± 1.2b	6.0 ± 1.9ab	6.6 ± 0.8a
20:1(n-11)	2.2 ± 0.5	1.3 ± 0.5	1.4 ± 0.5	1.6 ± 0.2
20:1(n-9)	4.3 ± 0.9a	2.4 ± 0.9b	2.7 ± 1.1b	2.4 ± 0.3b
20:1(n-7)	1.3 ± 0.4	2.0 ± 2.7	0.8 ± 0.6	0.2 ± 0.5
22:1(n-11 + 9)	2.5 ± 0.6	1.1 ± 1.3	ND	ND
20:2(n-9)	2.6 ± 0.3	1.8 ± 0.3	2.1 ± 0.7	2.2 ± 0.6
18:2(n-6)	3.5 ± 0.7	2.4 ± 0.9	2.7 ± 1.8	3.4 ± 0.3
20:2(n-6)	1.3 ± 0.4	1.1 ± 1.0	1.5 ± 1.1	1.6 ± 1.1
20:3(n-6)	2.3 ± 0.5	1.3 ± 0.9	1.8 ± 0.7	1.8 ± 0.3
20:4(n-6)	36.0 ± 7.9	22.0 ± 8.2	27.7 ± 10.4	28.4 ± 3.0
20:5(n-3)	ND	0.9 ± 0.7b	4.0 ± 1.4a	4.5 ± 1.1a
22:6(n-3)	0.8 ± 0.1b	2.9 ± 1.1b	10.2 ± 3.6ab	16.4 ± 8.2a
Total n-6	43.1 ± 9.6	26.8 ± 10.7	33.8 ± 11.7	35.2 ± 2.4
Total n-3	0.8 ± 0.1b	3.9 ± 1.8b	14.2 ± 5.1a	21.0 ± 7.4a
Total	152.2 ± 29.0	110.5 ± 31.7	162.5 ± 56.3	154.7 ± 14.1
Gd ml^{-1}	500	714 ± 25	2,144 ± 234	3,750 ± 181
Pm ml^{-1}	300,000	90,206 ± 7,062	51,426 ± 3,396	49,448 ± 2,670

Letters denote significant differences at the $P < 0.05$ level

Gd Gyrodinium dominans, *Pm Perkinsus marinus*, *ND* ≤0.2 μg

Table 4 Heterotroph biomass (#cells and μg carbon) and LCn-3EFA content in 300 ml cultures before and after 3 days grazing on the n-3 fatty acid-deficient protist *Perkinsus marinus* (mean ± SD, $n = 4$ replicate bottles). Heterotroph carbon contents (336 and 282 pg C $cell^{-1}$ for *O. marina* and *G. dominans*, respectively) were determined previously (Chu et al. 2008)

	Oxyrrhis marina		*Gyrodinium dominans*	
	Initial	Day 3	Initial	Day 3
Het cells ml^{-1}	500	5,210 ± 159	500	3,750 ± 181
Total Het cells ($\times 10^5$)	1.5	16.0 ± 0.5	1.5	11.0 ± 0.5
μg Het C	50.4	525.2 ± 16.0	42.3	317.2 ± 15.3
μg EPA	ND	1.9 ± 0.2	ND	4.5 ± 1.1
μg DHA	2.7 ± 0.5	17.4 ± 3.0	0.8 ± 0.1	16.4 ± 8.2

Het heterotrophic protist, *ND* ≤0.2 μg

of fatty acids containing a mixture of ^{13}C and ^{12}C were detected (Fig. 1). These results suggest that *O. marina* is capable of utilizing acetate exclusively for de novo fatty acid synthesis of some saturated, n-7 monounsaturated and n-3 polyunsaturated fatty acids and that *G. dominans* is not capable of metabolizing acetate incorporated in the culture medium.

Uptake, incorporation and metabolism of 18:3(n-3)-d4 encapsulated in GAMs by *O. marina* and *G. dominans*

When *G. dominans* and *O. marina* were fed GAMs containing Sudan Red-dyed olive oil, both protists ingested GAMs (Fig. 2). *G. dominans* and *O. marina* fed *D. teriolecta* supplemented with GAMs containing 18:3(n-3)-d4 proliferated

Table 5 Synthesis of uniformly labeled fatty acids by *Oxyrrhis marina* incubated in 300 ml medium containing 200 µg sodium 1,2 ^{13}C acetate ml^{-1}. *O. marina* cell density increased from 2,000 to 2,227 ± 90 cells ml^{-1}during the 24 h incubation (n = 3). Total fatty acid contents were determined by GC/FID. Percentage of uniformly ^{13}C-labeled fatty acids were determined by GC/MS using standard curves for molecular ions of each fatty acid component. Due to low recovery of 22:6(n-3) molecular ion incorporation of ^{13}C into this fatty acid was determined after hydrogenation to convert 22:6(n-3) to 22:0

FAME	µg all ^{12}C	µg all ^{13}C
14:0	1.6 ± 1.3	107.1 ± 47.2
16:0	13.5 ± 3.8	90.5 ± 28.2
18:0	3.0 ± 0.7	0.9 ± 0.3
16:1(n-9)	2.4 ± 0.5	ND
16:1(n-7)	ND	26.9 ± 8.4
18:1(n-9)	ND	5.7 ± 1.2
18:1(n-7)	ND	29.0 ± 9.0
18:2(n-6)	0.6 ± 0.1[a]	[a]
18:3(n-3)	0.4 ± 0.1[a]	[a]
20:5(n-3)	0.7 ± 0.1[a]	[a]
22:6(n-3)	5.9 ± 0.1	14.7 ± 2.6
Total	43.6 ± 10.8	274.8 ± 92.2

[a] Levels were too low for determination of ^{13}C incorporation by GC/MS

ND ≤0.2 µg

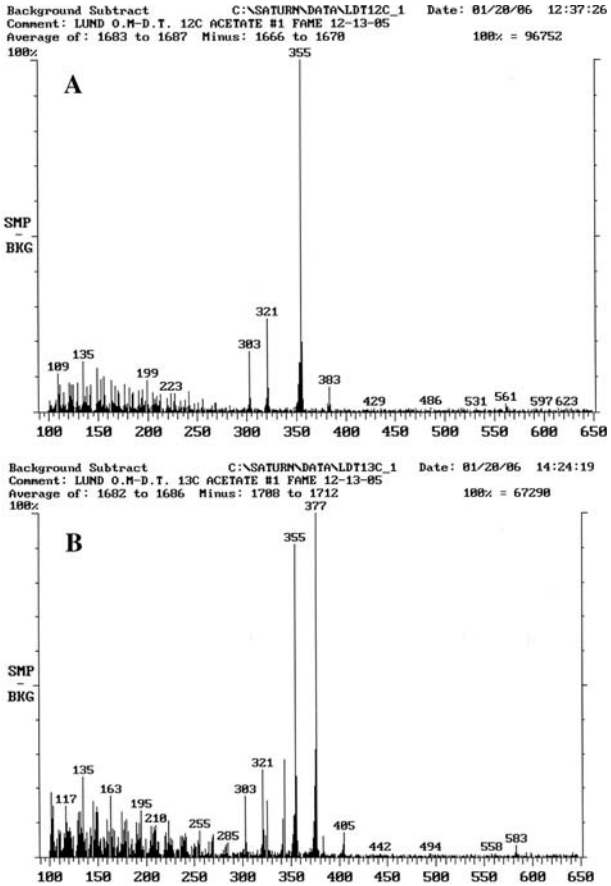

Fig. 1 GC/MS spectra of 22:0 FAME produced by hydrogenation of 22:6(n-3) FAME from *Oxyrrhis marina* cultures incubated with ^{12}C sodium acetate (**a**), or ^{13}C sodium acetate (**b**) for 24 h. The sample from ^{12}C acetate-incubated cells (**a**), has a molecular ion for hydrogenated 22:6(n-3) (converted to 22:0) of 355 Da (M+H). This corresponds to 22:6(n-3) containing only ^{12}C atoms. The sample from ^{13}C acetate-incubated cells (**b**), has two molecular ions for hydrogenated 22:6(n-3). One molecular ion is 355 Da (M+H) corresponding to 22:6(n-3) containing only ^{12}C atoms while the other molecular ion is 377 Da (M+H) corresponding to 22:6(n-3) containing only ^{13}C atoms

over the 2 days of the experiment. Cell density of *G. dominans* cultures increased from an initial cell concentration of 500–1,321 ± 273 cells ml^{-1} by day 2 (Table 6). *O. marina* cultures increased from an initial concentration of 500–2,404 ± 240 cells ml^{-1} (Table 6). *G. dominans* and *O. marina* cultures fed GAMs containing unlabeled 18:3(*n*-3) also increased in cell density, to 1,527 ± 224 cells ml^{-1} and 2,750 ± 156 cells ml^{-1} for *G. dominans* and *O. marina*, respectively. Replication of heterotrophs was associated with consumption of *D. tertiolecta* prey. By day two nearly 75% of the *D. tertiolecta* in *G. dominans* cultures and more than 99% of *D. tertiolecta* in *O. marina* cultures were consumed (Table 6). Ingested GAMs were also clearly visible in both heterotroph species in sample aliquots used to determine cell densities via light microscopy.

Results of GC/FID analysis of FAMEs derived from *G. dominans* and *O. marina* cultures collected on GF/F filters indicated that 9–10% of the 18:3(n-3)-d4 contained in the GAMs were recovered in *G. dominans* and *O. marina* (Table 6). Comparisons of GC/FID chromatograms of FAMEs derived from cultures of *G. dominans* and *O. marina* fed the 18:3(n-3)-d4 GAMs with those fed the unlabeled GAMs revealed two peaks present in the deuterated samples, but absent from the unlabeled samples (Fig. 3). These peaks were identified by relative retention time as 16:3(n-3)-d4 and 14:3(n-3)-d4 (Table 6), which are believed to be metabolites of 18:3(n-3)-d4, since these two peaks were absent in FAME derived from the 18:3(n-3)-d4 GAM and the 18:3(n-3) GAM. GC/FID analyses of the polyunsaturated fatty acid fractions obtained by preparative argenation TLC also contained these two metabolite peaks and removed a smaller peak (17:1) co-eluting with 16:3(n-3)-d4. This further confirms that they were polyunsaturated fatty acids. These FAME fractions were then converted to saturated FAMEs by hydrogenation. Later GC/MS analysis of the hydrogenated FAMEs identified 14:0-d4 FAME (mol. wt. = 246) and 16:0-d4 FAME (mol. wt. = 274) present in both *G. dominans* and *O. marina* fed the 18:3(n-3)-d4 GAMs. Only non-labeled 14:0 FAME (mol. wt. = 242) and 16:0 FAME (mol. wt. = 270) were detected in the hydrogenated FAMEs derived from cultures fed GAMs containing unlabeled 18:3(n-3). No 20:5(n-3) or 22:6(n-3) containing the deuterated label were detected by GC/FID or GC/MS in

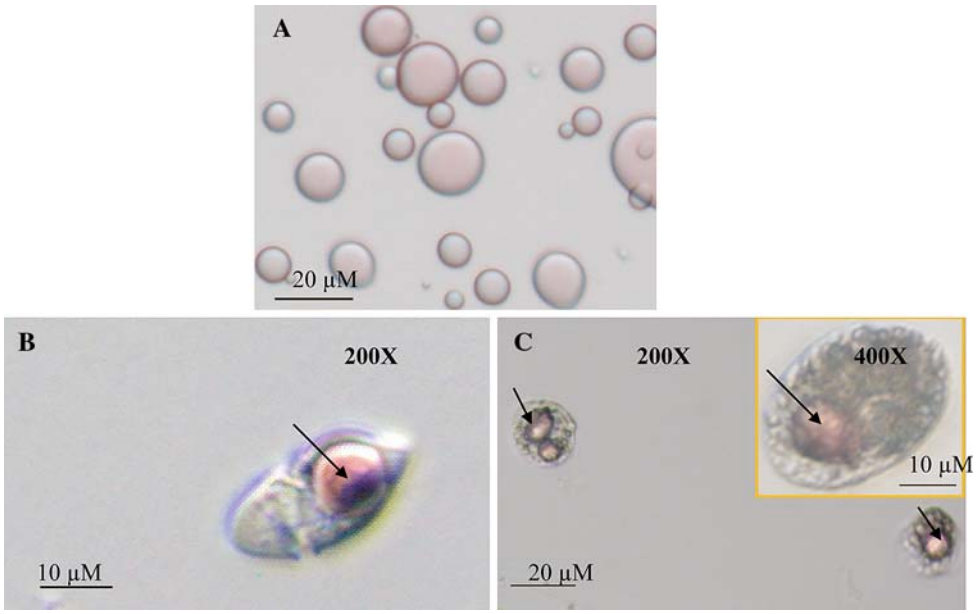

Fig. 2 Gelatin–acacia microcapsules (GAMs) containing oil stained with Sudan Red (×200) (**a**). Ingestion (see *arrow*) of GAMs by *Gyrodinium dominans* (×200) (**b**). Ingestion (see *arrows*) of GAMs by *Oxyrrhis marina* (×200 and ×400) (**c**)

Table 6 Incorporation and metabolism of 18:3(n-3)-d4 by 300 ml cultures of *Gyrodimium dominans* and *Oxyrrhis marina* after 2 days feeding *D. tertiolecta* and 18:3(n-3)-d4 GAMs. Initial concentrations of the heterotrophs *G. dominans* and *O. marina* were 500 cells ml^{-1} and the density of their prey, *D. tertiolecta*, was 50,000 cells ml^{-1}. All values are mean ± SD ($n = 4$)

	Gyrodinium dominans		*Oxyrrhis marina*	
	µg	Percent	µg	Percent
Total 18:3(n-3)-d4 contained in GAMs that were added to feed the protists	500.28 ± 19.94		1000.57 ± 39.89	
18:3(n-3)-d4 recovered with protists on GF/F	48.95 ± 7.88	9.79 ± 1.57	93.87 ± 3.05	9.38 ± 0.30
22:6(n-3)-d4	ND	ND	ND	ND
20:5(n-3)-d4	ND	ND	ND	ND
14:3(n-3)-d4	0.92 ± 0.12	0.18 ± 0.02	7.22 ± 0.31	0.72 ± 0.03
16:3(n-3)-d4	0.55 ± 0.08	0.11 ± 0.01	1.18 ± 0.17	0.12 ± 0.02
Heterotrophs ml^{-1}	1,321 ± 273		2,404 ± 240	
D. tertiolecta remaining (%)	25.2 ± 6.2		<1	

ND ≤0.2 µg or ≤0.1%

FAMEs derived from *G. dominans* and *O. marina* cultures fed 18:3(n-3)-d4 GAMs. These results suggest that both *G. dominans* and *O. marina* ingested GAMs and catabolized 18:3(n-3)-d4, but did not elongate and desaturate 18:3(n-3)-d4 to EPA or DHA.

Discussion

Microzooplankton occupy an important position in the microbial loop, because they are capable to modify the biochemical composition of autotrophs prior to transfer to the next trophic level. The ability of heterotophic protists to upgrade low nutritional quality food sources by producing the LCn-3EFAs, EPA, and DHA, seems to be one of the factors for the subsequent growth and reproduction improvement of the metazoan zooplankton, copepods and *Daphinia magna* (Klein-Breteler et al.1999; Tang and Taal 2005; Veloza et al. 2006; Bec et al. 2006). The phenomenon of "trophic upgrading" by various heterotrophic protist species in both the marine and freshwater systems has been reported and its benefit and ecological implication in aquatic food webs has been extensively discussed (e.g., Klein-Breteler et al.1999; Bec et al. 2003, 2006; Broglio et al. 2003; Tang and Taal 2005; Veloza et al. 2006). However, despite the potential ecological importance of LCn-3EFA production by marine and fresh water heterotrophic protists, the mechanism(s) employed by these microzooplankton to convert algal biomass and other substrates into LCn-3EFAs remain unclear. This area of research is largely uninvestigated and very few species have been characterized. What little is known involves primarily parasitic

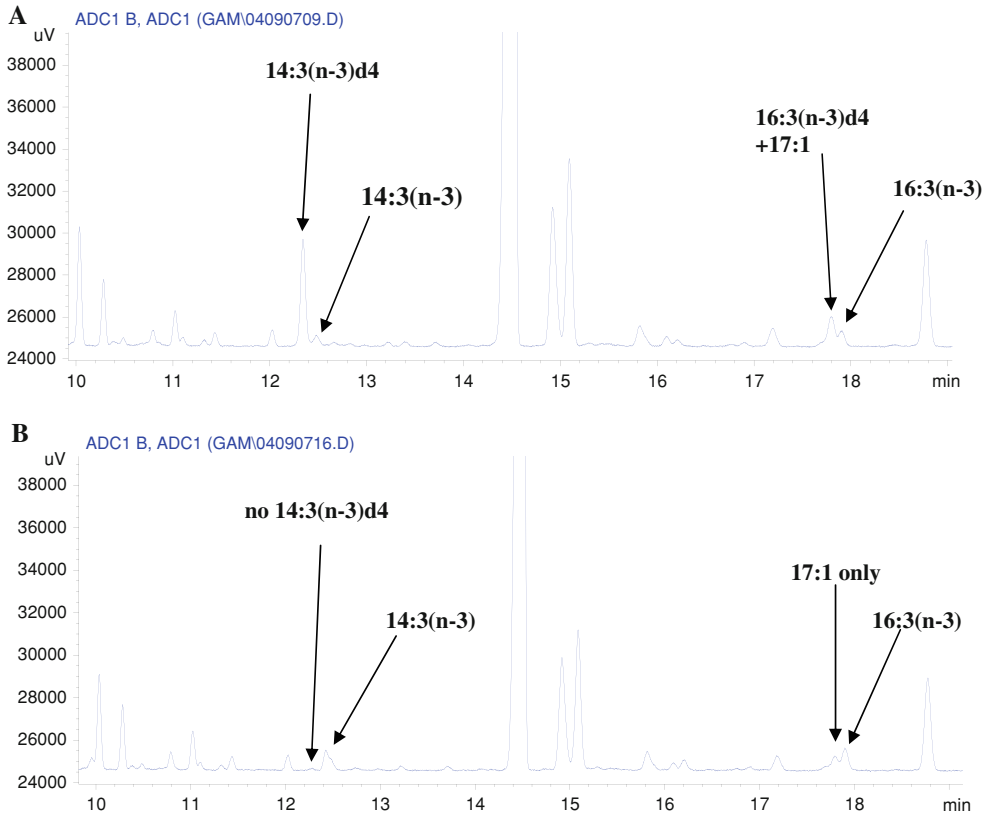

Fig. 3 GC/FID chromatograms of FAME from *Oxyrrhis marina* fed 18:3(n-3)-d4 GAMs (**a**), and *O. marina* fed 18:3(n-3) GAMs (**b**)

species and protists grown as osmotrophs (Metz et al. 2001; Ratledge 2004). Only one publication reported the incorporation of ^{14}C into EPA and DHA in a marine zooflagellate, *Bodo* sp. and a marine ciliate, *Euplotes crassus* after incubation with ^{14}C-labeled acetate for 24 h (Zhukova and Kharlamenko 1999). However, this technique does not discriminate between de novo synthesis and elongation/desaturation of precursor fatty acids. This study only recorded the distribution of ^{14}C in EPA and DHA, but did not clearly demonstrate the de novo synthesis of EPA and DHA and the approach does not allow quantitation of the "actual and absolute" production of EPA and DHA as we demonstrated in the present study. Understanding the dynamics and underlying mechanisms of carbon repackaging and "essential nutrient" upgrading is critical to determining the LCn-3EFA contribution in pelagic marine ecosystems since all higher trophic levels may depend on this production as their sole source of LCn-3EFAs at times of bloom dominated by algal species deficient of LCn-3EFAs. Heterotrophic protist production of LCn-3EFA may also be important when the primary producer standing stock during non-bloom period is dominated by pico- and nano-plankton, which are too small to be efficiently grazed by calanoid copepods and other zooplankton even though they may contain LCn-3EFA and LCn-3EFA precursors.

"Essential fatty acid upgrading" is believed to occur through selective bioaccumulation of LCn-3EFAs and/or elongation and desaturation of dietary LCn-3EFA precursors rather than de novo synthesis, based on results derived from feeding experiments employing either the LCn-3EFA-deficient alga (*D. tertiolecta*) which contains high level of precursors to EPA and DHA, or algae (e.g., *R. salina*) containing both EPA and DHA and precursors to them (Klein-Breteler et al. 1999; Broglio et al. 2003; Veloza et al. 2006). This is not the case, however, for the two heterotrophic protists tested in the present study. The cumulative evidence from the present study strongly suggests that both *O. marina* and *G. dominans* synthesize EPA and DHA de novo. This conclusion is supported by the findings that: (1) both *O. marina* and *G. dominans* synthesized DHA when fed a prey (the parasitic protist, *P. marinus*) deficient of n-3 fatty acids, (2) both *O. marina* and *G. dominans* catabolized 18:3(n-3)-d4 incorporated in GAMs to 16:3(n-3)-d4 and 14:3(n-3)-d4, but did not elongate/desaturate it to any C20 or C22 n-3 fatty acids, and (3) *O. marina*, though not *G. dominans* produces uniformly ^{13}C-labeled DHA when incubated with ^{13}C acetate.

Most eukaryotes utilize the aerobic fatty acid synthetic pathway for the production of LCn-3EFAs, using either type I or type II fatty acid synthases. In the aerobic pathway

18:0 is desaturated to 18:1(n-9) (Δ9 desaturase), then 18:2(n-6) (Δ12 desaturase), 18:3(n-3) (Δ15 desaturase), and 18:4(n-3) (Δ6 desaturase). An elongase converts 18:4 (n-3) to 20:4(n-3) which is then desaturated (Δ5) to form 20:5(n-3). In lower eukaryotes, 20:5(n-3) is elongated to 22:5(n-3), then desaturated (Δ4) to make 22:6(n-3) (Pereira et al. 2003; Qui 2003; Uttaro 2006). Use of this pathway for LCn-3EFA synthesis is characterized by: (1) the presence of n-3 intermediates such as 18:3(n-3), 18:4(n-3), 20:4(n-3), and 22:5(n-3) and (2) the utilization of dietary derived n-3 intermediates for LCn-3EFA production. Since no n-3 fatty acids other than EPA and DHA were detected when *O. marina* and *G. dominans* were fed the n-3 fatty acid deficient prey *P. marinus*, and 18:3(n-3)-d4 encased in GAMs was catabolized by both heterotrophs, but not elongated and desaturated to make EPA and DHA, it would appear that neither *O. marina* nor *G. dominans* utilizes the aerobic pathway for LCn-3EFA synthesis.

An anaerobic pathway for the synthesis of LCn-3EFAs by marine protists and marine bacteria has recently been described that utilizes polyketide synthases (PKS) instead of fatty acid synthases (Metz et al. 2001; Wallis et al. 2002; Uttaro 2006). Only 30 of the approximately 4,000 known species of dinoflagellates have so far been shown to produce polyketides (Rein and Barrone 1999). Of these polyketide producers a few species have been demonstrated to utilize the PKS pathway for the production of fatty acids (Napier 2002; Uttaro 2006). For example, the dinoflagellate *Crypthecodinium cohnii* produces >35% DHA with no 18C PUFAs when cultured as an osmotroph (Ratledge 2004) and has been demonstrated to use polyketide pathways to synthesize DHA using 2-C substrate such as acetate as building blocks (De Swaaf et al. 2003). The marine bacterium, *Shewanella* spp. synthesizes both EPA and DHA using PKS pathway (Metz et al. 2001). The presence of a PKS system involved in synthesis of DHA and EPA was also noted in the marine plant protistan parasites *Schizchytrium* sp. and *Ulkenia* sp. (Ratledge 2004; Uttaro 2006). These reports suggest that certain protist species from a wide variety of different groups utilize a PKS system for LCn-3EFA synthesis. But the prevalence of PKS use is not at all well characterized and the biochemical processes involved in the PKS system in synthesizing of LCn-3EFAs have not been completely elucidated.

Although the exact mechanism whereby LCn-3EFAs are synthesized using a PKS system is currently not understood, organisms utilizing a PKS system for the synthesis of LCn-3EFAs differ from those using the aerobic pathway in that they lack the series of elongases and desaturases used to synthesize LCn-3EFAs via the aerobic pathway (Metz et al. 2001; Wallis et al. 2002; Uttaro 2006). Also, one characteristic of the fatty acid composition of organisms utilizing a PKS system for the synthesis of LCn-3EFAs is that only very small amounts of shorter chain length n-3 fatty acids are present (Ratledge 2004). This feature has been attributed to these intermediates being tightly bound to the PKS in a manner that prevents their release as free fatty acyl-CoAs (Ratledge 2004). One very relevant related aspect to the observation that PKS binds fatty acyl-CoA intermediates very tightly is that this also prevents exogenous fatty acids from being utilized as substrates for LCn-3EFA synthesis. Both of these features, lack of n-3 fatty acid intermediates and inability to utilize dietary fatty acid precursors to make LCn-3EFAs are present in *O. marina* and *G. dominans*. Also, neither of these dinoflagellates elongates and desaturates dietary n-6 fatty acids (Tables 2 and 3), yet they proliferate and increase culture LCn-3EFA content when fed n-3 fatty acid-deficient prey (Table 4). Furthermore, they catabolize, but do not elongate and desaturate 18:3(n-3)-d4 encapsulated in GAMs (Table 6). Thus, it is unlikely that these two protists synthesize EPA and DHA via elongation and desaturation of their precursors, 18:3n-3 and 18:4n-3, derived from dietary sources. Additionally, similar to what has been found in the dinoflagellate *C. cohnii*, one especially interesting characteristic of the fatty acids produced from ^{13}C acetate by *O. marina* is that they are uniformly labeled (Fig. 1, Table 5). Apparently, ^{13}C acetate is directly converted to ^{13}C acetyl CoA via acetate thiokinase and the ^{13}C acetyl CoA is then used for fatty acid synthesis. However, whether *O. marina* and *G. dominans* use PKS system for LCn-3EFA synthesis remains to be confirmed and how uniformly ^{13}C-labeled DHA is made is unclear.

The findings that *O. marina* can utilize acetate provided in the medium to synthesize DHA and that both *O. marina* and *G. dominans* can produce LC n-3EFAs from n-3 fatty acid-deficient prey provide further proof of the ability of heterotrophic protists to use diverse substrates in addition to algae as a carbon source for LCn-3EFA production. This has potential implications for the mass and potential sources of substrates that could be used for LCn-3EFA synthesis in the marine environment. If heterotrophic protists were only able to synthesize LCn-3EFAs from shorter chain n-3 fatty acid precursors then algal production of these precursors would limit total LCn-3EFA production at the algae-heterotroph interface. However, the results from the present study suggest that any organic carbon source that can be utilized by a heterotroph may be converted to LCn-3EFAs. Furthermore, although previously it had been shown that certain heterotrophic protists as intermediate prey enhance the nutritional value of low food quality prey, by "upgrading" their biochemical constituents such as LCn-3EFAs and sterols for subsequent use by higher trophic levels, the ecological relevance of this process remains unclear. However, our very recent findings (Chu et al. 2008) suggest that under some conditions certain heterotrophic

protists have the potential to contribute significant amounts of LCn-3EFAs to the food webs and alter the DHA:EPA ratio of the diet to higher trophic levels. Both *O. marina* and *G. dominans* were found to contain high levels of DHA relative to three representative species of algae that contain it (47.6 ± 3.7 and 38.4 ± 3.6 μg DHA mg C^{-1} for *O. marina* and *G. dominans*, respectively, vs. a range of 2.1–17.9 μg DHA mg C^{-1} for the algal species *Chaetoceros calcitrans*, *Tetraselmis suecica* and *R. salina*) (Chu et al. 2008). Even when the heterotrophic protist DHA contents are normalized to μg DHA produced per mg algal C consumed, both *O. marina* and *G. dominans* produced quantities of DHA equal to or higher than the DHA content (μg DHA mg C^{-1}) of *C. calcitrans*, *T. suecica* and *R. salina* (22.3 ± 6.4 and 16.5 ± 1.1 μg DHA mg algal C ingested^{-1} for *O. marina* and *G. dominans*, respectively) (Chu et al.2008). These results suggest that consumption of algae by heterotrophic protists may potentially increase the amount of DHA available to zooplankton compared to direct consumption of autotrophic algae by zooplankton, even when the algae are not LCn-3EFA deficient.

The differences in substrate utilization between *O. marina* and *G. dominans* is notable and of particular interest in regard to the potential importance of heterotrophic protists as producers of LCn-3EFAs in the marine environment. *O. marina* has previously been cultured as an osmotroph under axenic conditions (Droop 1970; Droop and Pennock 1971), so the finding that this species can utilize DOM in the form of acetate for growth and fatty acid production is not in itself surprising. However, the ecological implications of this ability raise the possibility that LCn-3EFAs produced by heterotrophic protists can be made from carbon originating from sources other than autotrophic algae. If DOM, POM, bacteria, and algae can all be utilized by at least some heterotrophs to produce LCn-3EFAs, then the pool of carbon that can be utilized to produce these compounds is greater than previously suspected. On the other hand, *G. dominans* was apparently unable to utilize acetate in the medium for growth or fatty acid synthesis and thus, the carbon pool it can utilize for LCn-3EFA production may be limited to algal prey. Clearly, the issue of what carbon sources can be utilized for LCn-3EFA production by heterotrophic protists is more complex than previously acknowledged.

In summary, both *O. marina* and *G. dominans* do not utilize dietary fatty acid intermediates to synthesize LCn-3EFA. Thus, they do not directly "upgrade" LCn-3EFA precursors present in algal prey to LCn-3EFAs as previously believed. Rather, both species synthesize LCn-3EFAs de novo to produce a fatty acid profile that is typical of protists that utilize an anaerobic PKS system for LCn-3EFA synthesis. The two dinoflagellate species differ, however, in that *O. marina* uses acetate from the medium to synthesize uniformly labeled saturated and monounsaturated fatty acids and DHA, while *G. dominans* appears to be incapable of utilizing DOM and is dependent on capturing particulate prey. Our findings suggest that heterotrophic protists may play a more important role in the production of LCn-3EFAs in the marine pelagic ecosystem than has previously been acknowledged. Further study of the fatty acid synthetic capabilities of greater variety of marine heterotrophic protists is necessary to more clearly define their ecological importance in regulating carbon transfer efficiency and LCn-3EFA production at the phytoplankton–zooplankton interface.

Acknowledgments This study was supported by OCE, NSF (award #:0525899). The authors are grateful for Jennifer A. Podbesek's help in culturing the protist and algal cultures. Contribution no. 2939 by the Virginia Institute of Marine Science, College of William and Mary.

References

Bec A, Desvilettes C, Vera A, Lemarchand C, Fontvielle D, Bourdier G (2003) Nutritional quality of a freshwater heterotrophic dinoflagellate: trophic upgrading of its microalgal diet for *Daphnia hyaline*. Aquat Microb Ecol 32:203–207. doi:10.3354/ame032203

Bec A, Martin-Creuzburg D, von Elert E (2006) Trophic upgrading of autotrophic picoplankton by heterotrophic nanoflagellate *Paraphysomonas* sp. Limnol Oceanogr 51:1699–1707

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Boechat IG, Adrian R (2005) Biochemical composition of algivorous freshwater ciliates: you are not what you eat. FEMS Microbiol Ecol 53:393–400. doi:10.1016/j.femsec.2005.01.005

Broglio E, Jonasdottir SH, Calbet A, Jakobsen HH, Saiz E (2003) Effect of heterotrophic versus autotrophic food on feeding and reproduction of the calanoid copepod *Acartia tonsa*: relationship with prey fatty acid composition. Aquat Microb Ecol 31:267–278. doi:10.3354/ame031267

Christie WW (1989) Gas chromotography and lipids: a practical guide. Oily Press, Ayr

Chu F-LE, Webb KL, Hepworth DA, Casey BB (1987) Metamorphosis of larvae of *Crassostrea virginica* fed microencapsulated diets. Aquaculture 64:185–197. doi:10.1016/0044-8486(87)90324-3

Chu F-LE, Lund ED, Podbesek JA (2008) Quantitative significance of n-3 essential fatty acid contribution by heterotrophic protists and its ecological implication in marine pelagic food webs. Mar Ecol Prog Ser 354:85–95. doi:10.3354/meps07215

De Swaaf ME, De Rijk TC, Van der Meer P, Eggink G, Sijtsma L (2003) Analysis of docosahexaenoic acid biosynthesis in *Crypthecodinium cohnii* by ^{13}C labeling and desaturase inhibitor experiments. J Biotechnol 103:21–29. doi:10.1016/S0168-1656(03)00070-1

Dolan JR (1991) Microphagous ciliates in mesohaline Chesapeake Bay waters: estimates of growth rates and consumption by copepods. Mar Biol (Berl) 111:303–309. doi:10.1007/BF01319713

Droop MR (1970) Nutritional investigation of phagotrophic protozoa under axenic conditions. Helgolander Wiss Meersunters 20:272. doi:10.1007/BF01609905

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

Fessenden L, Cowles TJ (1994) Copepod predation on phagotrophic ciliates in Oregon coastal waters. Mar Ecol Prog Ser 107:103–111. doi:10.3354/meps107103

Kates M (1972) Techniques of lipidology; isolation analysis and identification of lipids. American Elsevier, NY

Klein-Breteler WCM, Schogt N, Baas M, Schouten S, Kraay GW (1999) Trophic upgrading of food quality by protozoans enhancing copepod growth: role of essential lipids. Mar Biol (Berl) 135:191–198. doi:10.1007/s002270050616

Lund ED, Chu F-LE, Soudant P, Harvey E (2007) *Perkinsus marinus*, a protozoan parasite of the eastern oyster, has a requirement for dietary sterols. Comp Biochem Physiol A 146:141–147. doi:10.1016/j.cbpa.2006.10.004

Martin-Creuzburg D, Bec A, von Elert E (2005) Trophic upgrading of picocyanobacterial carbon by ciliates for nutrition of *Daphnia magna*. Aquat Microb Ecol 41:271–280. doi:10.3354/ame041271

Marty Y, Delaunay F, Moal J, Samain J-F (1992) Changes in the fatty acid composition of *Pecten maximus* (L.) during larval development. J Exp Mar Biol Ecol 163:221–234. doi:10.1016/0022-0981(92)90051-B

Metcalfe LD, Schmitz AA (1961) The rapid preparation of fatty acid esters for gas chromatographic analysis. Anal Chem Wash 33:363. doi:10.1021/ac60171a016

Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M et al (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science 293:290–293. doi:10.1126/science.1059593

Napier JA (2002) Plumbing the depths of PUFA biosynthesis: a novel polyketide synthase-like pathway from marine organisms. Trends Plant Sci 7:51–54. doi:10.1016/S1360-1385(01)02191-4

Pereira SL, Leonard AE, Mukerji P (2003) Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. Prostaglandins Leukot Essent Fatty Acids 68:97–106. doi:10.1016/S0952-3278(02)00259-4

Qui X (2003) Biosynthesis of docosahexaenoic acid (DHA, 22:6–4, 7, 10, 13, 16, 19): two distinct pathways. Prostaglandins Leukot Essent Fatty Acids 68:181–186. doi:10.1016/S0952-3278(02)00268-5

Ratledge C (2004) Fatty acid biosynthesis in microogranisms being used for single cell oil production. Biochimie 86:807–815. doi:10.1016/j.biochi.2004.09.017

Rein KS, Barrone J (1999) Polyketides from dinoflagellates: origins, pharmacology, and biosynthesis. Comp Biochem Physiol B 124:117–131. doi:10.1016/S0305-0491(99)00107-8

Soudant P, Chu FLE (2001) Lipid class and fatty acid composition of the protozoan parasite of oysters, *Perkinsus marinus* cultivated in two different media. J Eukaryot Microbiol 48:309–319. doi:10.1111/j.1550-7408.2001.tb00319.x

Tang K, Taal M (2005) Trophic modification of food quality by heterotrophic protists: species-specific effects on copepod egg production and egg hatching. J Exp Mar Biol Ecol 318:85–98. doi:10.1016/j.jembe.2004.12.004

Tang K, Jakobsen HH, Visser AW (2001) *Phaeocystis globosa* (Prymnesiophyceae) and the planktonic food web: feeding, growth, and trophic interactions among grazers. Limnol Oceanogr 46:1860–1870

Uttaro AD (2006) Biosynthesis of polyunsaturated fatty acids in lower eukaryotes. IUBMB Life 58:563–571. doi:10.1080/15216540600920899

Veloza AJ, Chu FLE, Tang KW (2006) Trophic modification of essential fatty acids by heterotrophic protists and its effects on the fatty acid composition of the copepod *Acartia tonsa*. Mar Biol (Berl) 148:779–788. doi:10.1007/s00227-005-0123-1

Wallis JG, Watts JL, Browse J (2002) Polyunsaturated fatty acid synthesis: what will they think of next? Trends Biochem Sci 27:467–473. doi:10.1016/S0968-0004(02)02168-0

Zeldis J, James MR, Grieve J, Richards L (2002) Omnivory by copepods in the New Zealand subtropical frontal zone. J Plankton Res 24:9–23. doi:10.1093/plankt/24.1.9

Zhukova NV, Kharlamenko VI (1999) Sources of essential fatty acids in marine microbial loop. Aquat Microb Ecol 17:153–157. doi:10.3354/ame017153