



## Growth phase significantly decreases the DHA-to-EPA ratio in marine microalgae

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**Abstract** Microalgae are the principal producers of long-chain polyunsaturated fatty acids (LC-PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in marine ecosystems. Algae are used in aquaculture systems as direct or indirect feed for zooplankton, filter-feeding mollusks and larval stages of crustaceans and fish. Therefore, it is necessary to select nutrient-rich strains, with high levels of EPA and/or DHA, preferably during the stage of rapid growth. During the course of algal growth (exponential to stationary phase), many microalgal species accumulate lipids, especially triacylglycerols. However, relatively little is known about the effect of growth phase on LC-PUFA accumulation. In the present study, absolute and relative EPA and DHA levels of seven representative species of marine microalgae were determined during different growth phases in batch culture. Four species (*Phaeodactylum tricornutum*, *Thalassiosira weissflogii*, *Thalassiosira pseudonana* and *Rhodomonas salina*) accumulated fatty acids during growth. In all these species, intracellular EPA levels were higher during the late stationary growth phase than during exponential growth. In contrast, an increase in DHA content was not observed and therefore the DHA-to-EPA ratio was significantly lower in late stationary phase cultures. These results can be used to improve the nutritional value of microalgae cultivated for application in marine aquaculture systems.

**Keywords** Aquaculture · Growth phase · Fatty acids · LC-PUFAs · Phytoplankton

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## Introduction

Due to their high nutritional value, microalgae are important feed sources in aquaculture systems (Patil et al. 2005). Cultivated marine algae can be used directly, as live feed for (larval stages of) bivalves and crustaceans, or indirectly, as food for zooplankton such as rotifers which, in turn, are used to feed crustaceans or small fish larvae (Muller-Feuga 2000; Chauton et al. 2014). Besides this, several microalgal species are currently being investigated by the aquaculture industry as fish meal or fish oil replacement in the diet of commercially farmed fish (Kousoulaki et al. 2015; Sprague et al. 2015; Sørensen et al. 2016). Marine algae are key organisms in the production of essential long-chain polyunsaturated fatty acids (LC-PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These *n*-3 PUFAs are necessary for optimal nutrition and stress tolerance of marine fish, especially at the larval and juvenile stages (Khozin-Goldberg et al. 2011, and references therein). Most fatty acids in algal cells are present as part of membrane lipids (e.g., phospho- or glycosyl-glycerides) or as part of storage lipids, mainly triacylglycerols (TAGs), in the cytosol (Thompson 1996). LC-PUFA content and composition may show significant differences between and within algal classes. Each algal class has roughly its own fatty acid composition, and the EPA–DHA content between algal classes is highly variable (Guschina and Harwood 2006; Lv et al. 2010; Boelen et al. 2013). For a cost-effective production of nutrient-rich marine microalgae, with high levels of EPA and/or DHA, it is necessary to select highly productive strains while optimizing cultivation conditions. Besides taxon-specific EPA and DHA variability, intracellular LC-PUFA content may be influenced by growth conditions, such as temperature, irradiance and CO<sub>2</sub> concentration (e.g., Jiang and Gao 2004; Pal et al. 2011; Boelen et al. 2013). During the course of batch growth or when grown under stress, many microalgal species accumulate lipids, especially TAGs (Hu et al. 2008). For example, when cultured under nitrate or silicate starvation, TAGs accumulated up to 14–18 % of total dry weight in the marine diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricorutum* (Yu et al. 2009). Although most of the LC-PUFAs can be found in structural membrane lipids (Roessler 1990), LC-PUFAs could be partitioned to or deposited in TAGs during the stationary phase of growth (Khozin-Goldberg et al. 2002; Tonon et al. 2002; Guihéneuf and Stengel 2013). In three of the four species investigated by Tonon et al. (2002) (*Nannochloropsis oculata*, *T. pseudonana* and *Pavlova lutheri*), this resulted in a higher cellular EPA content. Tonon et al. (2002) also reported accumulation of DHA in *T. pseudonana* and *P. lutheri*, although the incorporation into TAGs was significantly lower and accumulation started later during the course of batch growth. In other studies, EPA or DHA content per algal biomass was reduced or not significantly affected by culture age or nutrient limitation (Klein Breteler et al. 2005; Hsiao and Blanch 2006; Gong et al. 2013; Nalder et al. 2015).

The aim of this study was to investigate intracellular accumulation of the essential *n*-3 LC-PUFAs EPA and DHA during different growth phases. Therefore, absolute and relative EPA and DHA levels of seven representative species of marine microalgae were determined during growth in batch culture. The implications of our results for the nutritional value of marine microalgae and thereby their application in aquaculture systems will be discussed.

## Materials and methods

### Experimental setup

Seven species of marine algae were selected, representing different taxonomic groups (Table 1). The selected species were known to have a high EPA and/or DHA content and were often already used in aquaculture. The cultures were obtained from the Roscoff Culture Collection (RCC, Roscoff, France) and the National Center for Marine Algae and Microbiota (NCMA, formerly CCMP, Maine, USA). The cultures were grown in a climate room at 20 °C in *f/2* enriched (Guillard and Ryther 1962) filter-sterilized seawater adjusted to a salinity of 35 ‰. The species were subjected to a light–dark cycle of 16:8 h (L:D), with a photon flux density of 90  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Before the start of the experiment, stock cultures were acclimated for at least 2 weeks to the culture conditions in a semi-continuous mode. The cultures were cultured in triplicate in Fernbach flasks with a working volume of approximately 0.5 L. Cell counts and photosystem II (PSII) fluorescence parameters were determined regularly to define growth phase and maximum quantum efficiency of PSII. PSII fluorescence measurements are widely used to recognize stress conditions of the photosynthetic apparatus. A decline in maximum quantum efficiency of PSII often occurs after the exponential phase of growth, when nutrients become limited. Samples for fatty acid analysis and algal biovolume were collected during exponential growth (EXP), at the end of the exponential growth phase or the beginning of the stationary phase (STAT) and after at least 5 days after the beginning of the stationary phase, usually after 14 days of batch growth (LATE STAT). To be able to measure true inter- and intraspecific differences, taking into account differences in total fatty acid content relative to algal biomass, the absolute amounts of EPA and DHA were determined, using algal biovolume as biomass unit. Algal biovolume was calculated from cell counts and cell size measurements. Cell numbers were determined using a Coulter XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA) as described by van de Poll et al. (2005). To calculate the cell volume of the algae, samples of about 1.5 mL of culture were analyzed using an inverted microscope. The sizes of 50 cells were measured, and cell volume was calculated according to stereometric formulas as given in Hillebrand et al. (1999).

### PAM fluorometry

PSII fluorescence parameters were measured with a pulse-amplitude-modulated chlorophyll fluorometer (Water-PAM; Heinz Walz GmbH, Germany). Samples (5 mL) were

**Table 1** Details of the investigated species

Strain	Class	Strain#	Abbreviation
<i>Phaeodactylum tricornutum</i>	Bacillariophyceae	CCMP2558	PT
<i>Thalassiosira weissflogii</i>	Bacillariophyceae	CCMP1049	TW
<i>Thalassiosira pseudonana</i>	Bacillariophyceae	RCC950	TP
<i>Skeletonema costatum</i>	Bacillariophyceae	RCC70	SC
<i>Emiliana huxleyi</i>	Prymnesiophyceae	CCMP2112	EH
<i>Isochrysis galbana</i>	Prymnesiophyceae	RCC179	IG
<i>Rhodomonas salina</i>	Cryptophyceae	CCMP1319	RS

dark-adapted for 15 min and placed in a custom-made cuvette ( $2 \times 2$  cm). Basal fluorescence ( $F_0$ ) was measured under weak measuring light, and maximal fluorescence ( $F_m$ ) was determined after a saturating light pulse (0.8 s,  $4000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). The maximum quantum efficiency of PSII was calculated as  $F_v/F_m = (F_m - F_0)/F_m$  (Maxwell and Johnson 2000).

### Fatty acid analysis

Lipids were extracted using a modified Bligh and Dyer extraction as described by Guckert et al. (1988). Subsequently, the lipids were subjected to a mild alkaline methanolic transesterification (White et al. 1979) to produce fatty acid methyl esters (FAMES). Samples of 100 mL of algal culture were centrifuged, and the remaining pellets were freeze-dried for 48 h. The pellets were extracted for 18 h in a single-phase solvent system of 3.5 mL chloroform, 7.5 mL methanol and 2.8 mL 50 mM phosphate buffer (pH 7.4), and a known amount of nonadecanoic methyl ester (Sigma) was added as an internal standard. The samples were transferred to 50-mL centrifuge tubes, and 3.5 mL of chloroform and 3.5 mL of ultrapure Milli-Q water (Millipore) were added. After centrifugation for 5 min, the lower chloroform phase was dried under  $\text{N}_2$  and the remaining lipids were resuspended in 1 mL of a methanol/toluene mixture (1:1, v/v). One milliliter of 0.2 N MeOH–KOH was added, and the mixture was incubated for 15 min at  $37^\circ\text{C}$ . After neutralizing to pH 6 with 1 M acetic acid, 2 mL of chloroform and 2 mL of Milli-Q water were added. After centrifugation, the chloroform phase with the FAMES was recovered and the nonpolar FAMES were separated from the polar compounds over a small  $\text{Al}_2\text{O}_3$  column using dichloromethane (DCM) as eluent. The FAMES were analyzed on a Hewlett-Packard 5890 gas chromatograph equipped with both a flame ionization detector (FID) and a HP 5972 mass spectrometer detector (MSD). Samples of  $5 \mu\text{L}$  were injected using an HP 6890 autosampler at  $60^\circ\text{C}$  on a Restek RTX 1701 ( $60 \text{ m} \times 0.25 \text{ mm}$ , film thickness  $0.25 \mu\text{m}$ ) column. The temperature was held at  $60^\circ\text{C}$  for 1 min and then increased at a rate of  $10^\circ\text{C min}^{-1}$  until  $180^\circ\text{C}$  and then to a final temperature of  $250^\circ\text{C}$  at  $5^\circ\text{C min}^{-1}$ , which was maintained for 15 min. The injection temperature was  $250^\circ\text{C}$ , and the detector temperatures were both  $280^\circ\text{C}$ . Long-chain fatty acids (number of carbons  $\geq 14$ ) were identified from mass spectra and retention times by comparison with those of PUFA No. 1 standard mixture (Matreya LLC, USA). Quantification of fatty acids was done by integration of appropriate peak areas calibrated with the known concentration of the added internal standard.

### Statistical analysis

Differences in  $F_v/F_m$ , fatty acid content or DHA-to-EPA ratio between growth phases and species were analyzed by a two-way analysis of variance (ANOVA) followed by a simple main effects analysis when there was a significant interaction. Significant ( $p < 0.05$ ) differences among growth phases were further analyzed by pairwise comparisons using the Šidák adjustment for multiple comparisons. Values of relative EPA and DHA content (% of total fatty acids) were square-root-transformed prior to analysis. All statistical analyses were performed using IBM SPSS Statistics (version 22) software (IBM Corporation, Armonk, NY, USA).

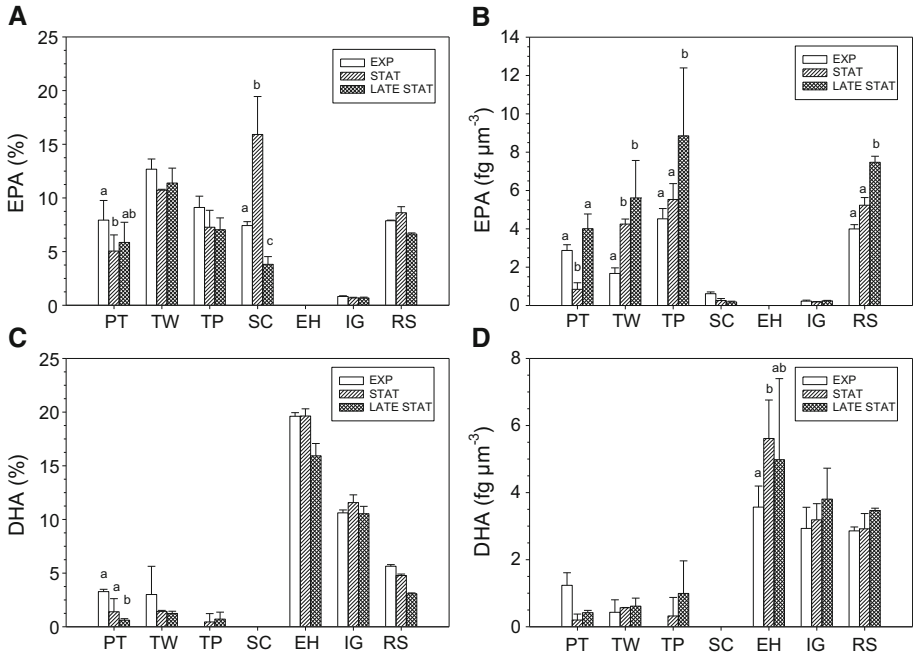
## Results

Relative (% of total fatty acid) and absolute (normalized to biovolume) EPA and DHA content showed large variability between the tested species (Table 2; Fig. 1). The diatoms (Bacillariophyceae) showed high EPA and a relatively low DHA percentages. In contrast, the two prymnesiophytes were characterized by a high DHA and a low EPA content. *Rhodomonas salina*, a representative cryptophyte species, showed both high EPA and DHA percentages compared to the other species. Although EPA percentages in *S. costatum* were comparable with the other diatoms (9 % of total fatty acids on average), absolute levels of EPA normalized to biovolume were low (<1 fg  $\mu\text{m}^{-3}$ ). DHA percentages were highest in *E. huxleyi* (up to 20 % of total fatty acids), while absolute levels were

**Table 2** Statistical results of two-way ANOVA for effects of growth phase and algal species on relative (% of total fatty acids), absolute (normalized to biovolume) EPA and DHA content, total fatty acid content (fg  $\mu\text{m}^{-3}$ ), maximum quantum efficiency of PSII ( $F_v/F_m$ ) and DHA-to-EPA ratio

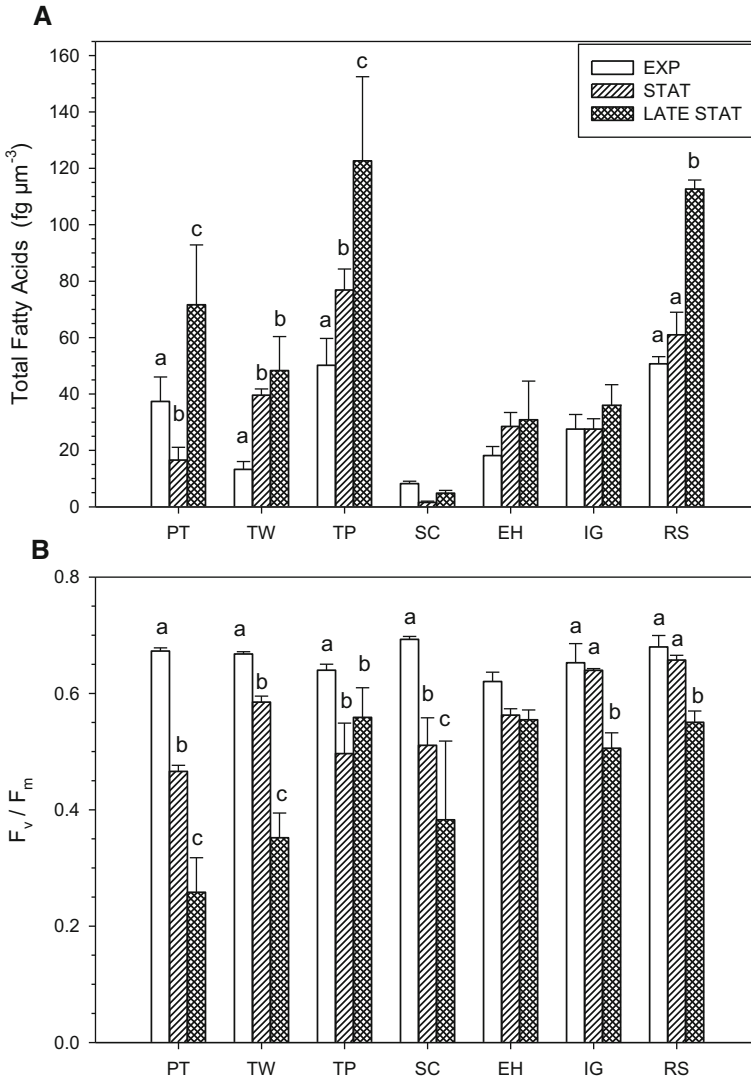
Effect on	Source of variation	df	MS	F	p
EPA %	Species	6	12.221	190.2	<b>&lt;0.001</b>
	Growth phase	2	0.444	6.9	<b>0.003</b>
	Species × growth phase	12	0.577	9.0	<b>&lt;0.001</b>
	Residual	42	0.064		
DHA %	Species	6	18.798	97.9	<b>&lt;0.001</b>
	Growth phase	2	0.180	0.9	0.400
	Species × growth phase	12	0.542	2.8	<b>0.006</b>
	Residual	42	0.192		
EPA	Species	6	61.606	69.3	<b>&lt;0.001</b>
	Growth phase	2	19.174	21.6	<b>&lt;0.001</b>
	Species × growth phase	12	4.245	4.8	<b>&lt;0.001</b>
	Residual	42	0.89		
DHA	Species	6	30.479	58.8	<b>&lt;0.001</b>
	Growth phase	2	1.158	2.2	0.120
	Species × growth phase	12	0.799	1.5	0.148
	Residual	42	0.519		
Total fatty acids	Species	6	6924.594	67.9	<b>&lt;0.001</b>
	Growth phase	2	5849.409	57.3	<b>&lt;0.001</b>
	Species × growth phase	12	840.981	8.2	<b>&lt;0.001</b>
	Residual	42	101.932		
$F_v/F_m$	Species	6	0.026	15.5	<b>&lt;0.001</b>
	Growth phase	2	0.230	138.6	<b>&lt;0.001</b>
	Species × growth phase	12	0.018	10.6	<b>&lt;0.001</b>
	Residual	42	0.002		
DHA-to-EPA ratio	Species	2	0.321	67.7	<b>&lt;0.001</b>
	Growth phase	2	0.158	33.3	<b>&lt;0.001</b>
	Species × growth phase	4	0.014	3.0	0.052
	Residual	16	0.005		

Statistically significant *p* values (*p* < 0.05) are printed in bold



**Fig. 1** Relative (% of total fatty acids) (A, C) and absolute (normalized to biovolume) (B, D) EPA and DHA content in seven species of marine algae during different growth phases. Values represent averages ( $\pm$ SD) of three replicate cultures. Different letters (per species) represent significant differences at  $p < 0.05$  among growth phases according to the analysis of simple main effects. EXP = exponential phase, STAT = stationary phase, LATE STAT = late stationary phase. PT = *Phaeodactylum tricorutum*. TW = *Thalassiosira weissflogii*. TP = *Thalassiosira pseudonana*. SC = *Skeletonema costatum*. EH = *Emiliania huxleyi*. IG = *Isochrysis galbana*. RS = *Rhodomonas salina*

comparable with *I. galbana* and *R. salina* (between 3 and 6  $\text{fg } \mu\text{m}^{-3}$ ). Also, the total fatty acid content varied significantly between species (Table 2; Fig. 2a). The lowest amounts (between 1.6 and 8.2  $\text{fg } \mu\text{m}^{-3}$ ) were found in *S. costatum*, while the highest total fatty acid content ( $122.6 \pm 29.8 \text{ fg } \mu\text{m}^{-3}$ ) was measured in *T. pseudonana* during the late stationary phase of growth. For all species, the maximum quantum efficiency of PSII ( $F_v/F_m$ ) decreased during growth (Fig. 2b), indicating that the cultures were subjected to stress during the late stationary phase. The interactive effects of species  $\times$  growth phase were highly significant (Table 2), indicating that the effect of growth phase on total fatty acid content was not the same for all species. Out of the seven species tested, four species (*P. tricorutum*, *T. weissflogii*, *T. pseudonana* and *R. salina*) showed a significantly enhanced total fatty acid content in the late stationary phase as compared to the exponential growth phase (Fig. 2a). Also, the absolute amount of EPA in these species was higher during the late stationary growth phase than during exponential growth, although this effect was not statistically significant for *P. tricorutum* (Fig. 1b). In *P. tricorutum*, absolute EPA and total fatty acid content initially decreased with growth, but both levels were significantly higher at the late stationary growth phase as compared to the stationary growth phase. Highest absolute EPA levels were found in *T. pseudonana* and *R. salina* (8.9 and 7.5  $\text{fg } \mu\text{m}^{-3}$ , respectively). The highest increase in EPA during batch growth was found in *T. weissflogii*. In this species, the absolute EPA content was 3.4 times higher during the late



**Fig. 2** Total fatty acid content (fg μm<sup>-3</sup>) (A) and maximum quantum efficiency of PSII ( $F_v/F_m$ ) (B) of seven species of marine microalgae during different growth phases. Values represent averages (±SD) of three replicate cultures. Different letters (per species) represent significant differences at  $p < 0.05$  among growth phases according to the analysis of simple main effects. EXP = exponential phase, STAT = stationary phase, LATE STAT = late stationary phase. PT = *Phaeodactylum tricornutum*. TW = *Thalassiosira weissflogii*. TP = *Thalassiosira pseudonana*. SC = *Skeletonema costatum*. EH = *Emiliania huxleyi*. IG = *Isochrysis galbana*. RS = *Rhodomonas salina*

stationary growth phase than during exponential growth. On the other hand, absolute levels of DHA during the late stationary phase were not significantly higher than levels in exponentially growing cultures (Table 2; Fig. 1d). DHA-to-EPA ratios were calculated for species with initial EPA and DHA levels both higher than 1 % (Table 3). In all these species, the DHA-to-EPA ratio decreased significantly during growth. The highest DHA-

to-EPA ratio was measured in *R. salina* during exponential growth ( $0.72 \pm 0.02$ ). The highest decrease was observed in *P. tricorutum*. In this species, the DHA-to-EPA ratio was almost four times lower during the late stationary growth phase than during exponential growth.

## Discussion

In this study, we focused on the accumulation of the essential *n*-3 LC-PUFAs EPA and DHA in batch cultures of marine algae. Although many studies have demonstrated accumulation of (neutral) lipids in starved cultures of microalgae, relatively little was known about the effect of growth phase on EPA and DHA accumulation. Our study confirms previous results that the absolute amounts of EPA and DHA, but also the total fatty acid content varies considerably between species (Volkman et al. 1989; Viso and Marty 1993; Tonon et al. 2002; Mansour et al. 2005; Patil et al. 2007; Boelen et al. 2013). Consequently, high interspecific variability in their nutritional value exists.

Four out of the seven investigated microalgal species accumulated fatty acids during batch growth. For *E. huxleyi*, *I. galbana* and *S. costatum*, no significant accumulation of lipid compounds could be demonstrated. Possibly, other photosynthetic products (e.g., carbohydrates) accumulated in these species when entering the nutrient-depleted stage (Harrison et al. 1990; Granum et al. 2002).

In all lipid-accumulating species, absolute cellular EPA levels were higher during the late stationary growth phase than during exponential growth. The relative abundances (% of total fatty acids) of EPA in these algae remained relatively constant during the stage of growth, while total cellular fatty acid content was significantly increased. This could be an indication that at least part of the EPA is transferred into or present in TAGs, since in most algae stress-induced lipids are predominantly in the form of TAGs (Hu et al. 2008). It is hypothesized that microalgal TAG can serve as a depot for LC-PUFAs, which could be mobilized for the construction of chloroplastic membranes under sudden changes in environmental conditions (Cohen et al. 2000; Khozin-Goldberg et al. 2005). High proportions of LC-PUFAs present in TAGs could be an advantage when culturing microalgae to produce an alternative for fish oil (Tonon et al. 2002; Leu and Boussiba 2014).

The absolute amount of DHA, however, was not affected, and therefore, the DHA-to-EPA ratio in species containing DHA as well as EPA decreased significantly during growth. This implies that in these species, the accumulation of EPA and DHA seems to be regulated by two different mechanisms. This effect could have consequences for the nutritional quality, since the nutritional value of algae is dependent not only on the total

**Table 3** Effect of growth phase on DHA-to-EPA ratio in three species of marine microalgae. DHA-to-EPA ratios were calculated for species with initial EPA and DHA levels both higher than 1 % (relative to total fatty acids)

Strain	EXP	STAT	LATE STAT
<i>Phaeodactylum tricorutum</i>	$0.43 \pm 0.14^a$	$0.41 \pm 0.17^a$	$0.11 \pm 0.01^b$
<i>Thalassiosira weissflogii</i>	$0.35 \pm 0.00^a$	$0.13 \pm 0.01^b$	$0.11 \pm 0.01^b$
<i>Rhodomonas salina</i>	$0.72 \pm 0.02^a$	$0.56 \pm 0.05^b$	$0.46 \pm 0.02^b$

Values represent averages ( $\pm$ SD) of three replicate cultures. Different letters per row (species) represent significant differences at  $p < 0.05$  among growth phases according to the analysis of simple main effects. EXP = exponential phase, STAT = stationary phase, LATE STAT = late stationary phase



amount of EPA and/or DHA but also on the relative proportion of these LC-PUFAs (Brown 2002; Spolaore et al. 2006). For fish larval nutrition, DHA-to-EPA ratios of between 1 and 2 are advised (Rodríguez et al. 1998). For *R. salina*, which contains significant percentages of both DHA and EPA and is used as aquaculture feed (e.g., Gagné et al. 2010), this would imply that although the total cellular amount of fatty acids is higher, the nutritional value of this species is lower at the end of the growth phase.

Currently, several microalgal species are being investigated by the aquaculture industry as fish meal or fish oil replacement in the diet of commercially farmed fish (Kousoulaki et al. 2015; Sprague et al. 2015; Sørensen et al. 2016). Replacing the traditional EPA- and DHA-rich, but finite, marine ingredients, fishmeal and fish oil present a significant challenge for the aquaculture industry (Sprague et al. 2016). Promising fish oil alternatives are lipids from microalgae which can potentially grow at high growth rates and which can reach high maximum cell densities with high levels of EPA and/or DHA. Much research has been focused on heterotrophic DHA-producing species such as *Schizochytrium* (Kousoulaki et al. 2015; Sprague et al. 2015). However, Sprague et al. (2015) showed that the absence of EPA in *Schizochytrium*-based fish diet significantly impairs the overall nutritional value. Sørensen et al. (2016) investigated the potential use of the phototrophic EPA-rich *P. tricornutum* as a fish meal replacement in diet for Atlantic salmon. They showed that *P. tricornutum* can replace up to 6 % of the fish meal without adverse effects on nutrient digestibility, utilization of the feed and growth performance. In future studies, it would be useful to test mixtures of DHA-rich *Schizochytrium* and EPA-rich phototrophic algal species as alternative sources of EPA and DHA in aquafeeds. In our study, we showed that intracellular EPA levels in *P. tricornutum* and three other marine algal species can be further enhanced by optimizing the harvest time.

We conclude that in some marine algal species EPA accumulates during batch growth. The highest increase in EPA was measured in *T. weissflogii*, while highest levels were measured in *T. pseudonana* and *R. salina*. Absolute levels of DHA were not affected, which can have consequences for the nutritional value of these algae. This effect has to be taken into account when batch culturing microalgae as a food source in aquaculture systems.

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