frontiers in Marine Science

ORIGINAL RESEARCH
published: 12 March 2019
doi: 10.3389/fmars.2019.00095

Influence of Nitrogen Limitation on Lipid Accumulation and EPA and DHA Content in Four Marine Microalgae for Possible Use in Aquafeed

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Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: 06 November 2018
Accepted: 18 February 2019
Published: 12 March 2019

Citation:
Wang X, Fosse HK, Li K, Chauton MS, Vadstein O and Reitan KI (2019) Influence of Nitrogen Limitation on Lipid Accumulation and EPA and DHA Content in Four Marine Microalgae for Possible Use in Aquafeed. Front. Mar. Sci. 6:95. doi: 10.3389/fmars.2019.00095

Microalgae are regarded as a promising alternative that can replace fishmeal and fish oil in aquaculture. Under N-limitation, many microalgae species change their carbon storage patterns in favor of neutral lipids (NLs) mainly in the form of triacylglycerol (TAG), but fatty acids in polar lipids (PL) are nutritionally more available for fish than those esterified into NLs. In the present study, the effect of N-limitation on the lipid content and fatty acid profiles in different lipid classes of *Phaeodactylum tricornutum, Isochrysis aff. galbana clone* T-Iso, *Rhodomonas baltica,* and *Nannochloropsis oceanica* were investigated. The microalgae cells were cultivated by two different methods, batch and semi-continuous culture, to create strong and moderate N-limitation, and this in turn will significantly affect the biomass and lipid productivity. All four species accumulated lipids mainly in the form of TAG, in response to strong nitrogen limitation. *N. oceanica*, however, accumulated 51% of the dry weight as lipid in moderate nitrogen limitation and up to 87% of the fatty acid was in TAG. *Isochrysis* aff. *galbana* clone T-Iso was the only species where the fraction of polyunsaturated fatty acid (PUFA), especially the fraction of docosahexaenoic acid (DHA), increased with increasing nitrogen limitation. Total lipid productivity showed no increase in batch culture although stronger nitrogen limitation led to lipid accumulation. *P. tricornutum* had the highest eicosapentaenoic acid (EPA) content, while *N. oceanica* showed the highest EPA productivity due to the high content of lipid. The highest DHA productivity was found in *Isochrysis* aff. *galbana* clone T-Iso from moderate N-limitation, mainly due to the high biomass productivity. Based on the results from the current study, *N. oceanica* and T-Iso are two promising microalgae strains ass long-term sustainable sources of *n*-3 long chain -PUFAs under moderate N-limitation. As shown in the present study, increased lipid content in microalgal cells due to strong N-limitation induction may not increase the lipid productivity because biomass production is usually reduced. Therefore, a combination of approaches such as metabolic engineering, conditioning and selection may be needed to further increase the n-3 LC-PUFA productivity without substantial loss of biomass.

Keywords: nitrogen, cultivation conditions, marine microalgae, lipids, PUFAs, aquafeed

INTRODUCTION

Fish oils are used as a lipid source fish feed due to the high content of long-chain polyunsaturated fatty acids (LC-PUFA). However, the global supply of fish oils is limited and it cannot meet the future demands for aquaculture and human consumption (Salem and Eggersdorfer, 2015). As a result, alternative raw materials are increasingly being evaluated for use in aquafeed formulation (Chauton et al., 2014).

Many efforts have been made to replace fish oils with vegetable oils in formulated fish feed, but the major limit of using vegetable oils is their lack of n-3 LC-PUFA. Microalgae, on the other hand, are natural primary producers of n-3 LC-PUFA (Shah et al., 2018). The typical EPA-producing microalgae species include *Nannochloropsis* spp, *Monodus subterraneus*, *Nitzschia* spp., and the model diatom *P. tricornutum* (Wen and Chen, 2000; Lu et al., 2001; Hu and Gao, 2003; Yang et al., 2013). Whereas, the typical DHA-producing species include *Isochrysis galbana* (Reitan et al., 1994; Fidalgo et al., 1998) and the thraustochytrids *Aurantochytrium* spp. (Taoka et al., 2009) *Thraustochytrium* spp. and *Schizochytrium* spp. (Wang et al., 2018). Moreover, microalgae with high nutritional values are widely used in aquaculture, either for direct consumption for molluscs and shrimp, or indirectly through live feed for fish larvae (Reitan et al., 1997; Brown and Robert, 2002). Previous studies demonstrated that microalgae contain on average 30–40% protein, 10–20% lipids, and 5–15% carbohydrates (Brown et al., 1997; Reitan et al., 1997). Some species, for instance *Nannochloropsis* spp., contain as much as 37–60% (by DW) lipids and are identified as strong candidates for lipid production (Doan et al., 2011).

Phylogenetic affiliation to a large extent determines the potential lipid content and fatty acid profile (Cañavate et al., 2016), but biochemical composition of the algal biomass can be modulated by varying growth conditions (Reitan et al., 1994; Chen et al., 2011; Fakhry and El Maghraby, 2015; Vu et al., 2016). Research on nitrogen limitation has been conducted for decades, as it is one of the most important strategies to increase triacylglycerol (TAG) content (Chen et al., 2015). Under N-limitation, many microalgae species change their carbon storage patterns in favor of neutral lipids (NLs) mainly in the form of TAG (Reitan et al., 1994; Illman et al., 2000; Rodolfi et al., 2009). In *N. oceanica*, the lipid content doubled under N-depletion compared to N-repletion, and glycerolipids (mainly TAG) increased from 8.3 to 44.2% (Jia et al., 2015). The EPA content in TAG increased from 3.6 μmol g^{-1} DW under N-repletion to 21 μmol g^{-1} DW under N-limitation, and the accumulation of EPA was correlated with the degradation of EPA-containing membrane glycerolipids (Jia et al., 2015). The accumulation of TAG and changes in fatty acid profiles under N-limitation have also been observed in several diatoms, green algae and red algae (Rodolfi et al., 2009; Sharma et al., 2012; Valenzuela et al., 2012; Yang et al., 2013). However, these types of studies mainly focus on comparing nitrogen-deficient to nitrogen-sufficient conditions in one cultivation method (Breuer et al., 2012; Griffiths et al., 2012; Chen et al., 2015). Some studies investigated the lipid content in response to different nitrogen concentrations and temperature in batch culture (Converti et al., 2009; Fakhry and El Maghraby, 2015). The fatty acid profiles of NLs and polar lipids (PLs) have a great impact on the selection of cultivation and processing strategies (Meireles et al., 2003; Huang et al., 2013). For example, when algae are cultured for the supply of PUFAs, FAs in PLs are nutritionally more available for fish than those esterified into NLs (Guedes et al., 2010). There are few studies that focus on lipid accumulation under different degrees of N-limitation and the localization of n-3 LC-PUFA content in different lipid class.

In the present study, the effect of N-limitation on the lipid content and fatty acid profiles in different lipid class of *P. tricornutum*, *Isochrysis* aff. *galbana* clone T-Iso, *R. baltica,* and *N. oceanica* were investigated. The microalgae cells were cultivated by two different methods, batch and semi-continuous culture, to create strong and moderate N-limitation, and this in turn will significantly affect the biomass and lipid productivity. Because EPA and DHA are the two fatty acids with the highest value when considering microalgae as an alternative source to fish oil, the production and the localization of these fatty acids were also studied.

MATERIALS AND METHODS

Microalgae and Culture Medium

Four microalgae species (1) *Phaeodactylum tricornutum* CCMP 2561, (2) *Isochrysis* aff. *galbana* clone T-Iso CCAP 927/14, (3) *Rhodomonas baltica* NIVA-5/91, and (4) *Nannochloropsis oceanica* CCMP1779 were investigated in the present study. Stock cultures of *Phaeodactylum tricornutum* CCMP 2561 and *Rhodomonas baltica* NIVA-5/91 were from SINTEF Ocean, Trondheim, while *Isochrysis* aff. *galbana* clone T-Iso CCAP 927/14 and *Nannochloropsis oceanica* CCMP1779 were from NTNU, Center of Fisheries and Aquaculture. Prior to each experiment microalgae were grown in cell culture flasks with f/2 medium (Guillard, 1975) at 20°C for about 3 days. Seawater was filtered (0.22 μm) and autoclaved before use.

Cultivation Methods

To obtain micraolgae cells with strong and moderate N-limitation, we analyzed cells from late stationary phase (μ <0.05 μ_{max}) in batch culture (BT) and from steady state ($\mu = 0.5\ \mu_{max}$) in semi-continuous culture (SC).

In BT, preacclimated aliquots of *P. tricornutum,* T-Iso, *and R. baltica*-cultures were inoculated into 2.4 L modified f/2 medium (10% of the original amount of sodium nitrate and 25% more sodium phosphate than the original receipt) and divided into two 1.2 L glass cylinders (LWS 05, Inst. Getreideverarbeitung GmbH, Germany). In order to have enough samples for further analysis, *N. oceanica* were inoculated into 8 L of modified f/2 medium and divided into 8 cylinders. Cultures were constantly illuminated by artificial light, with an average light intensity of about 150 μmol photons $m^{-2}s^{-1}$ at the culture surface. The temperature was kept constant at 22°C. In the first 2 days of cultivation, filtered air was supplied to the cultures. From day 3, the filtered air was mixed with 650 ppm CO_2, and pH was thereafter maintained around 8.5. The supply of filtered air, together with magnetic

stirring ensured proper mixing in the cultures. Cultures were grown for 7 or 8 days, depending on species, until reaching the stationary phase.

In SC, about 100 mL stock cultures from BT were inoculated into the same cultivation system, with parallel cultures for *P. tricornutum,* T-Iso *and R. baltica,* and 8 replicates for *N. oceanica*. Algae in the SCs were grown at a specific growth rate equal to 0.5μ max by controlling the dilution rate. Daily dilution with harvesting at the same time each day started from day 4 or 5. Algal cells were collected as described for BT when the cultures reached a steady state phase at day 7 or 8. This was continued for about 20 days to collect a sufficient biomass for later analysis. The biomasses from all samplings were pooled together.

Harvesting

Algal cells were harvested by centrifugation at 10,000 g for 10 min at 4°C. The supernatant was discarded and cell pellets were transferred into 5 mL centrifuge tubes, rinsed with distilled water and centrifuged at 10,000 g (Jouan KR 22i centrifuge) for 10 min at 4°C. N_2 gas was added to cell pellets and these were then stored at −80°C for further analysis. Parallel cultures of each microalgae were pooled together during the centrifugation to obtain enough material for analyses.

Determination of Maximum Growth Rate and Dilution Rate for SC

About 5 mL cultures from each cylinder were sampled daily to follow the growth. Optical density (OD) was measured in a spectrophotometer (UviLine 9100, Schott® Instruments, Germany) at 750 nm. Cell numbers of *P. tricornutum,* T-Iso, and *R. baltica* were counted by coulter counter (Multisizer™ 3 Coulter Counter®, Beckman Coulter Inc., Miami, FL, USA) and *N. oceanica* was counted in Hemocytometer under microscope.

Maximum growth rates of the algae (μ_{max}) in early exponential phase were calculated (Reitan et al., 1994) and the dilution rate of SC was set at 50% of μ_{max}. In steady state dilution cultures, the growth rate was controlled by the dilution rate (Reitan et al., 1994).

Carbon and Nitrogen Analysis

For C and N analysis, exact volumes (10–15 mL) of the cultures were filtered through pre-combusted (480°C, 2 h) Whatman GF/C glass fiber filters and stored at −20°C. Before C and N analysis, filters were exposed to vapor of concentrated hydrochloric acid for 15 min to remove inorganic carbon. The filters were packed into tin capsules and dried at 60°C for 48 h. The analysis of C and N were done in an Elemental Combustion System CHNS-O (Costech ECS, model 4010, Costech International, Firenze, Italy).

Total Lipids and Fatty Acids Analysis

Total lipids were extracted by homogenizing cells in chloroform/methanol (2:1, v/v), filtering through a glass fiber filter before adding 0.88% potassium chloride to the mixture. After centrifugation at 1,640 g for 10 min, the hypophase was collected and evaporated under N_2. The total lipid content was then determined gravimetrically (Folch et al., 1957). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transesterification at 50°C for 16 h (Christie, 2003), and FAME was extracted and quantified with C19:0 as an internal standard by a gas chromatograph (AutoSystem XL, Perkin Elmer, Waltham, MA) with Total Chrom Version 6.3.1 software. The system was equipped with an auto-injector (1 μl, inlet temperature 250°C) and a flame ionization detector (FID, 280°C). The temperature program for the oven was 90°C for 1 min, then raised to 150°C at 30°C min^{-1} and finally raised to 225°C at 3°C min^{-1} and held for 7 min. Helium was used as the carrier gas, and the fused silica capillary column was coated with chemically bound polyethylene glycol (CP-Wax 52CB, 25 m × 0.25 mm i.d; Varian, Palo Alto, CA).

Triacylglycerols and Polar Lipids Analysis

Triacylglycerols (TAG) and polar lipids (PL) were separated on HPTLC silica gel 60 plates (10 × 10 cm, Merck KGaA Damstadt, Germany). Each plate was activated at 110°C for 1 h and 20 μl of the extracted lipid (10 mg mL^{-1} in chloroform/methanol 2:1, v/v) was applied as a 1 cm streak by a LINOMAT IV sprayer (CAMAG, Mutlenz, Switzerland). The plate with lipids was developed in hexane: diethylether: acetic acid (70:30:1, v/v) and visualized under UV light, after spraying with 1 mg mL^{-1} 2,7-dichlorofluorescein in methanol/water (95:5, v/v) containing 0.1 mg mL^{-1} BHT. Triacylglycerols and polar lipids were scraped into glass tubes and acid-catalyzed transesterification was performed as described above. The FAMEs were washed with 2% potassium bicarbonate, concentrated under N_2, and quantified by gas chromatograph as described above.

Calculation of Biomass and Lipid Productivity

The dry weight (DW) of the microalgae were calculated as:

$$DW = 2.45 * Carbon\ content \quad (1)$$

where 2.45 is a constant number (Huo et al., 2015).

The biomass productivity per day (P) of SC cultures were calculated as:

$$P = \frac{DW^* \Delta V}{culture\ volume} \quad (2)$$

where ΔV is the daily diluted volume and culture volume is the total volume of culture. Productivity in the BT cultures were calculated as the biomass at harvest divided by the number of cultivation days. The productivity value was further used to calculate lipid, EPA, and DHA productivity.

Statistical Analyses

Mean ± standard error of the mean (SEM) is presented, and differences in μ_{max} between different species were tested with one-way ANOVA and Tukey's multiple comparison test. Differences in means of N:C ratio, C and N $cell^{-1}$, total lipid content and productivity were tested for each species between two cultivation methods with a *t*-test in SPSS (IBM SPSS statistics 24). Means of fatty acid in the same species between two cultivation methods were tested with a *t*-test, and % fatty acid

was transformed to arcsin square root prior to *t*-test. Means of maximum specific growth rate (μ_{max} d^{-1}) between species were compared with one-way ANOVA followed by Tukey's multiple comparison tests. All tables were made in Excel 2016 and figures in SIGMA PLOT 14.0 (Systat Software Inc).

RESULT

Growth Parameters

Batch cultivation was conducted over 7 days for *P. tricornutum,* T-Iso, and *R. baltica*, and over 8 days for *N.oceanica* (**Figure 1**). All four microalgae species grew exponentially and entered the stationary phase before harvesting. Optical density was measured during the cultivation, and the measurements were linearly correlated with the growth in cell numbers of the cultures in all four microalgae species ($R^2 = 0.93–0.99$). For *R. baltica,* T-Iso, and *N.oceanica* the μ_{max} were found between day two and three, and for *P. tricornutum* between day three and four (**Table 1**). *P. tricornutum* had the highest μ_{max} and *N. oceanica* had the lowest ($p < 0.05$).

From day five or six, daily harvesting (according to 50% of μ_{max}) in the SC cultures was initiated, and the cell density was nearly the same from day to day (**Figure 1**). This shows a steady state growth, where the growth rate is determined by the dilution rate (D) and the cells are chemically equal from day to day.

Total Content of Carbon, Nitrogen, and Lipid

The N:C ratio of *P. tricornutum*, T-Iso and *N. oceanica* was 24–33% higher in SC than in late stationary phase in BT ($p < 0.05$). No significant difference was found for *R. baltica* ($p > 0.05$) (**Table 1**). The C content per cell was significantly higher in SC compared to BT for *R. baltica*, whereas it was 42% higher in BT for *N.oceanica* ($p < 0.05$). The N content per cell was 32–42% higher in SC than in BT for *P. tricornutum*, T-Iso and *R. baltica* ($p < 0.05$), while it was the same for *N.oceanica* ($p > 0.05$).

The highest lipid content was found in *N. oceanica* in SC, whereas *P. tricornutum* in SC had the lowest content of lipids (**Figure 2**). The content of total lipid increased by 58–96% in *P. tricornutum*, T-Iso, and *R. baltica* with increased N-limitation ($p < 0.05$). This was the opposite for *N. oceanica*, where the lipid content was 16% lower in BT compared to SC ($p < 0.05$).

Fatty Acid Composition

The FA composition varied between species (**Table 2**), but all four species in our study showed a high content of 16:0 (palmitic acid). *P. tricornutum* and *N. oceanica* had similar FA profiles, dominated by 14:0, 16:0, 16:1n-7, and 20:5n-3 (EPA). Together these FAs contributed with approximately 80 and 86% of total fatty acids in *P. tricornutum* and *N. oceanica*, respectively. In addition, *N. oceanica* had a high percentage of 18:1n-9 (oleic acid) and *P. tricornutum* had highest percentage of 20:5n-3 (EPA) among the four species. T-Iso and *R. baltica* had similar FAs profiles, with 14:0, 16:0, 18:1n-9, 18:2n-6, and 18:4n-3 as dominating fatty acids. They represented 63 and 74% of the total FAs, respectively. For *R. baltica*, the fatty acid 18:3n-3 was found at a high percentage, whereas T-Iso had a high percentage of 22:6 n-3.

For *P. tricornutum*, the percentage of monounsaturated fatty acid (MUFA) increased by 36%, whereas PUFA decreased by 43% in BT compared to SC ($p < 0.05$). In T-Iso the response

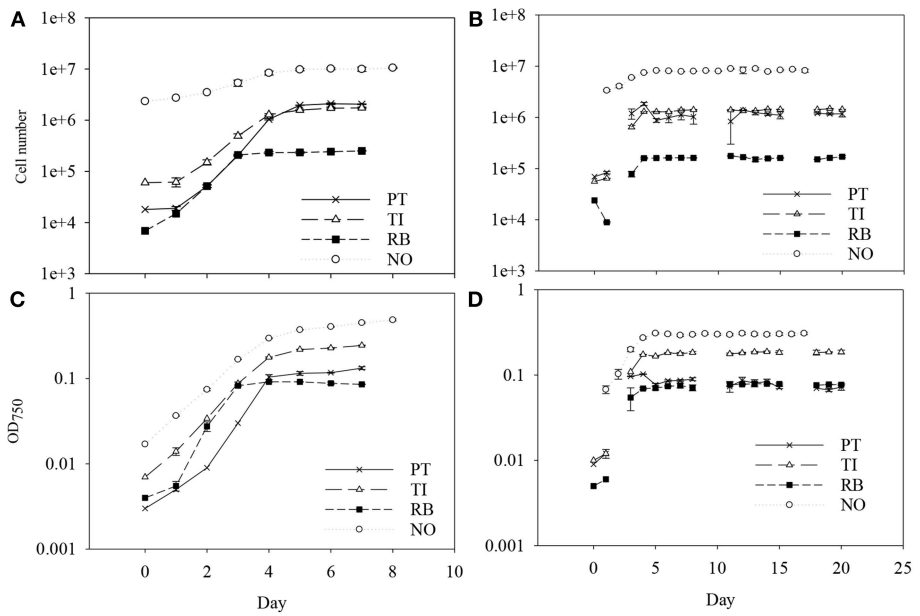

FIGURE 1 | The cell number and the optical density for batch culture **(A,C)** and for semicontinuous culture **(B,D)** of four marine microalgae species cultured under nitrogen limitation. Mean with SE bars. PT, *P. tricornutum*; TI, *Isochrysis aff. galbana* clone T-Iso; RB, *Rhodomonas baltica*; NO, *N. oceanica*.

TABLE 1 | Maximum specific growth rate (μ_{max} d^{-1}), N:C ratio, carbon, and nitrogen content per cell for four microalgae species from batch and semi-continuous culture.

	μ_{max}	N:C ugN mg C^{-1}		pg C $cell^{-1}$		pg N $cell^{-1}$	
	BT	BT	SC	BT	SC	BT	SC
P. tricornutum	1.63 ± 0.01[a]	71.5 ± 2.30	94.3 ± 0.74*	8.91 ± 0.15	9.92 ± 2.56	0.63 ± 0.02	0.90 ± 0.22*
T-Iso	1.19 ± 0.02[c]	68.3 ± 0.63	84.9 ± 0.19*	10.4 ± 1.28	10.8 ± 0.98	0.71 ± 0.08	0.94 ± 0.08*
R. baltica	1.40 ± 0.10[b]	80.2 ± 0.27	78.9 ± 1.92	57.6 ± 0.24	81.3 ± 1.44*	4.60 ± 0.04	6.38 ± 0.00*
N.oceanica	0.43 ± 0.01[d]	30.9 ± 0.26	41.1 ± 1.61*	5.68 ± 0.29	4.14 ± 0.20*	0.17 ± 0.01	0.17 ± 0.01

Mean ± SE. Means of maximum specific growth rate (μ_{max} d^{-1}) between species were compared with one-way ANOVA followed by Tukey's multiple comparison test. Letters represented the order of significant differences between the means: a > b > c > d. t-test were carried out for N:C ratio, carbon and nitrogen content per cell between BT and SC in the same species. Asterisks indicate significant difference.

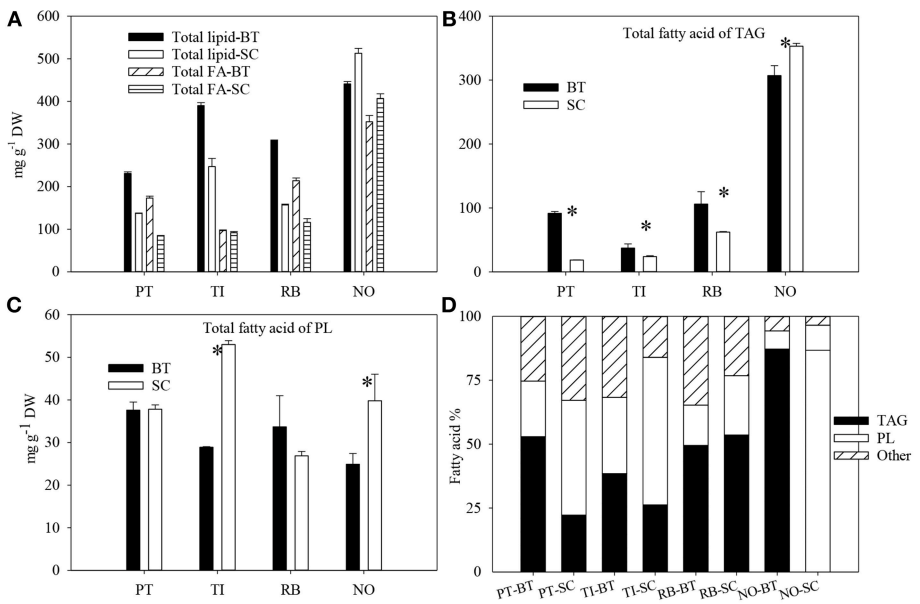

FIGURE 2 | The total lipid content and total fatty acid content **(A)**, total fatty acid content in triacylglycerol **(B)**, total fatty acid content in polar lipids **(C)**, and fatty acid distribution (%) in triacylglycerol, polar lipids and others **(D)** of four marine microalgae species cultured under nitrogen limitation, by batch (BT) and semi-continuous culture (SC). Mean with SE bars. Asterisk shows significant difference ($p < 0.05$). PT, *P. tricornutum*; TI, *Isochrysis aff. galbana* clone T-ISO; RB, *R. baltica*; NO, *N. oceanica*.

in distribution of fatty acids to strong N-limitation was: 16% decrease in SFA, 13% increase in PUFA, and no change in MUFA ($p < 0.05$). *N. oceanica* also showed a decrease in SFA and PUFA, of 8 and 12%, respectively, in BT compared to SC ($p < 0.05$). Opposite to T-Iso there was a 15% increase in MUFA ($p < 0.05$).

In *R. baltica,* SFA and MUFA increased by 18 and 39%, respectively, whereas PUFA decreased by 24% with increasing N-limitation ($p < 0.05$). The percentage of EPA decreased by 21–45% in *P. tricornutum*, *R. baltica,* and *N. oceanica*, whereas DHA increased by 16% in T-Iso with increasing N-limitation ($p < 0.05$).

Fatty Acid Composition in Triacylglycerols and Polar Lipids

The FA content of TAG in the four species were similar to that of total lipids, and the FA content was higher in BT compared to SC except for *N. oceanica* ($p < 0.05$) (**Figure 2**). The total fatty acids of polar lipids (PL) in T-Iso and *N. oceanica* decreased by 45 and 37% respectively ($p < 0.05$), with increased N-limitation, whereas it showed no changes in *R. baltica* and *P. tricornutum* ($p > 0.05$). The fatty acids were localized mainly in TAG in *P. tricornutum* and T-Iso in BT, and mainly in PL in SC. For *N. oceanica*, 86–87% of the fatty acids were localized in TAG both in BT and in SC.

The dominating fatty acids in TAG in *P. tricornutum* were 16:0 and 16:1n-7, and EPA was found at a low percentage (**Table 3**). The fatty acids 16:0, 18:1n-9, 18:2n-6, 18:4n-3, and 22:6n-3 were the dominating fatty acids of TAG in T-Iso, and only 18:4n-3 increased with increasing N-limitation ($p < 0.05$). The fatty acids 14:0, 16:0, 18:1n-9, 18:2n-6, 18:3n-3, and 18:4n-3 predominated the fatty acid profiles of TAG in *R. baltica*, and EPA was found in a low amount, similar to *P. tricornutum*. All n-3 PUFAs in *R. baltica* increased in BT ($p< 0.05$). *N. oceanica* had the highest fatty acid content in TAG among the

TABLE 2 | Fatty acid profiles (% of total fatty acids) of four microalgae species from batch and semi-continuous culture.

	P. tricornutum		T-Iso		*R. baltica*		*N.oceanica*	
	BT	**SC**	**BT**	**SC**	**BT**	**SC**	**BT**	**SC**
% OF TOTAL FATTY ACIDS								
C14:0	3.88 ± 0.02	6.28 ± 0.07*	10.3 ± 0.01	14.6 ± 0.02*	16.1 ± 0.08*	14.9 ± 0.02	6.37 ± 0.00	6.94 ± 0.04*
C16:0	31.6 ± 0.02*	26.4 ± 0.04	14.5 ± 0.11	15.6 ± 0.19*	23.6 ± 0.11*	19.4 ± 0.12	39.2 ± 0.01	42.5 ± 0.12*
C18:0	2.01 ± 0.01	1.91 ± 0.02	0.96 ± 0.02	1.16 ± 0.14	1.53 ± 0.05*	0.73 ± 0.04	1.19 ± 0.01	1.71 ± 0.15
22:00	nd	0.41 ± 0.01*	1.16 ± 0.00*	0.65 ± 0.00	nd	nd	nd	nd
∑SFA	37.7 ± 0.04	35.3 ± 0.01*	27.1 ± 0.14	32.1 ± 0.28*	41.5 ± 0.24*	35.2 ± 0.18	47.0 ± 0.01	51.5 ± 0.01*
16:1n-7	40.5 ± 0.00*	29.9 ± 0.06	1.89 ± 0.13*	1.27 ± 0.04	1.69 ± 0.02	2.60 ± 0.06*	34.5 ± 0.01*	30.8 ± 0.19
18:1n-9	1.21 ± 0.02*	0.70 ± 0.01	24.1 ± 0.08	24.3 ± 0.04	16.0 ± 0.02*	8.23 ± 0.13	10.6 ± 0.01*	8.48 ± 0.03
18:1n-7	0.88 ± 0.01*	0.49 ± 0.05	0.82 ± 0.01*	0.79 ± 0.00	2.08 ± 0.01	3.32 ± 0.00*	0.38 ± 0.00	0.36 ± 0.01
∑MUFA	42.6 ± 0.03*	31.1 ± 0.12	26.8 ± 0.06*	26.3 ± 0.00	19.8 ± 0.05*	14.2 ± 0.08	45.5 ± 0.01*	39.6 ± 0.15
18:2n-6	1.01 ± 0.01	1.13 ± 0.02*	6.22 ± 0.01	7.16 ± 0.03*	11.6 ± 0.05*	7.73 ± 0.06	0.68 ± 0.00	0.68 ± 0.00
20:3n-6	nd	1.05 ± 0.03*	nd	nd	nd	nd	nd	nd
20:4n-6	1.02 ± 0.21*	nd	nd	nd	0.71 ± 0.00	0.59 ± 0.04	1.63 ± 0.00*	1.35 ± 0.02
∑n-6	2.02 ± 0.20	2.18 ± 0.05	6.22 ± 0.01	7.16 ± 0.03*	12.3 ± 0.05*	8.32 ± 0.03	2.31 ± 0.00*	2.03 ± 0.01
18:3n-3	0.22 ± 0.00	nd	2.97 ± 0.01*	2.63 ± 0.01	10.1 ± 0.05	15.7 ± 0.01*	nd	nd
18:4n-3	0.76 ± 0.02	1.32 ± 0.01*	10.9 ± 0.03*	8.81 ± 0.04	7.36 ± 0.03	12.9 ± 0.00*	nd	nd
20:5n-3	9.56 ± 0.04	17.7 ± 0.13*	0.57 ± 0.06*	nd	4.21 ± 0.02	6.31 ± 0.02*	4.27 ± 0.00	5.43 ± 0.04*
22:6n-3	1.05 ± 0.00	2.38 ± 0.00*	15.2 ± 0.02*	13.1 ± 0.13	2.21 ± 0.02	3.83 ± 0.03*	nd	nd
∑n-3	11.5 ± 0.16	21.4 ± 0.15*	29.6 ± 0.04*	24.5 ± 0.08	23.9 ± 0.03	39.4 ± 0.05*	4.27 ± 0.00	5.43 ± 0.04*
∑PUFA	13.5 ± 0.03	23.6 ± 0.20*	35.8 ± 0.03*	31.7 ± 0.11	36.2 ± 0.08	47.7 ± 0.03*	6.59 ± 0.00	7.46 ± 0.05*

Fatty acid composition is expressed as percentage values of total fatty acids. Values represent Mean ± SE, n = 3. t-test were carried out for fatty acids in the same species. Asterisks indicate significant difference.

∑SFA, sum of saturated fatty acid; ∑MUFA, sum of mono unsaturated fatty acid; ∑PUFA, sum of polyunsaturated fatty acid; nd, not detected.

four species, with 16:0, 16:1n-7, and 18:1n-9 as dominating fatty acids.

The dominating fatty acids in PL in *P. tricornutum* were 16:0, 16:1n-7, and 20:5n-3, and they accounted for 62–68% of the total fatty acids in PL (**Table 4**). The fatty acid 16:0 remained the same, whereas 16:1n-7 and 20:5n-3 decreased by 11 and 16% ($p < 0.05$), respectively in BT. 14:0, 16:0, 18:1n-9, 18:4n-3, and 22:6n-3 fatty acids were the dominating fatty acids of PL in T-Iso, and they showed a slight decrease or remained the same in BT, except for the 14% increase in 18:1n-9 ($p < 0.05$). For *R. baltica*, a 51–77% decrease in all n-3-PUFAs was observed in BT ($p < 0.05$). 16:0, 18:0, 16:1n-7, 18:1n-9, 20:4n-6, and 20:5n-3 were the dominating fatty acids of PL in *N. oceanica*, and all fatty acids had no significant differences in BT compared to SC ($p > 0.05$).

Biomass and Lipid Productivity

The microalgae species that had the highest biomass productivity was *N. oceanica* at the late stationary phase with strong N-limitation (**Figure 3**). The major difference between the two cultivation methods was found in *R. baltica*. The biomass productivity decreased by 60–67% ($p < 0.05$) in BT in all microalgae species except for *N. oceanica*.

The highest lipid production was obtained in *N. oceanica* in SC and lowest in *P. tricornutum* and *R. baltica* in SC, 7.51 and 1.43 and 1.50 mg $L^{-1}d^{-1}$, respectively. The lipid productivity decreased in BT, compared with SC in all microalgae species except *N. oceanica*. The production of EPA doubled in *P. tricornutum* and *R. baltica* and increased by 31% in *N. oceanica* in SC ($p < 0.05$). The productivity of DHA doubled in *P. tricornutum* and T-Iso, and increased by three times in *R. baltica* in SC ($p < 0.05$). *N. oceanica* had the highest EPA productivity and T-Iso had the highest DHA productivity, both were in SC.

DISCUSSION

Microalgae may be a valuable feed raw material because of their natural content of PUFAs, but phylogeny and cultivation conditions determine the cellular lipid content. Lipid accumulation correlates with some limiting factors (often N), which in turn may result in a lower biomass yield (and overall lower lipid yield). Production of essential fatty acids from microalgae is therefore a compromise between maximizing the lipid content by modulation of growth conditions without lowering biomass production, or a matter of harvesting cells at the right moment.

The total lipid content varied between the four marine microalgae species tested here, and between the two cultivation methods. Among the four species, *N. oceanica* had the highest lipid content under moderate N-limitation, representing the high interest in lipid production. *P. tricornutum*, *R. baltica,* and T-Iso had higher lipid content in batch culture, indicating that these three species accumulate lipids mainly when strong N-limitation stops cell division (Breuer et al., 2012; Griffiths et al., 2012). However, this trend was the opposite for *N. oceanica*. The lipid

TABLE 3 | Fatty acid profiles in triacylglycerol (TAG) of four microalgae species cultured under nitrogen limitation, by batch (BT), and semi-continuous culture (SC).

% of fatty acid	*P. tricornutum*		T-Iso		*R. baltica*		*N.oceanica*	
	BT	SC	BT	SC	BT	SC	BT	SC
14:0	3.24 ± 0.59	5.74 ± 0.70*	3.37 ± 0.28	5.12 ± 1.22*	11.5 ± 3.85	14.7 ± 1.21	5.73 ± 0.05	6.57 ± 0.07*
16:0	34.1 ± 1.14	36.0 ± 0.36*	12.1 ± 0.75	15.0 ± 0.33*	24.8 ± 0.06	21.2 ± 0.33*	41.1 ± 0.19	44.6 ± 0.00*
18:0	1.07 ± 0.09	1.47 ± 0.23*	1.22 ± 0.58	2.16 ± 0.01	5.82 ± 0.59*	0.71 ± 0.04	1.74 ± 0.63	2.90 ± 0.29
20:0	nd	nd	nd	nd	nd	nd	0.11 ± 0.01	0.19 ± 0.01*
22:0	nd	nd	0.44 ± 0.03	0.78 ± 0.05	nd	nd	nd	nd
∑SFA	38.8 ± 1.28	43.5 ± 0.19*	16.43 ± 0.27	23.2 ± 0.77*	42.1 ± 4.51	36.6 ± 0.93	48.8 ± 0.39	54.4 ± 0.23*
16:1n7	45.6 ± 1.07*	40.9 ± 1.05	1.65 ± 0.48	1.53 ± 0.63	2.31 ± 0.27	3.31 ± 0.09*	34.6 ± 0.44*	31.0 ± 0.12
18:1n9	1.83 ± 0.17*	1.22 ± 0.41	29.0 ± 3.92	38.9 ± 1.27*	20.1 ± 1.72*	11.2 ± 0.49	10.3 ± 0.05*	8.77 ± 0.03
18:1n7	1.35 ± 0.18*	0.64 ± 0.11	1.06 ± 0.44	0.87 ± 0.39	1.39 ± 0.05	2.50 ± 0.06*	0.53 ± 0.03	0.53 ± 0.01
∑MUFA	48.8 ± 1.00*	43.0 ± 0.27	32.3 ± 4.10	41.6 ± 0.99*	23.8 ± 1.50*	17.0 ± 0.61	45.4 ± 0.46*	40.3 ± 0.10
18:2n6	0.99 ± 0.04*	0.36 ± 0.04	7.99 ± 1.37	12.5 ± 0.42*	12.4 ± 1.18*	9.29 ± 0.33	0.54 ± 0.01*	0.45 ± 0.02
20:2n6	nd	nd	0.58 ± 0.14	0.57 ± 0.02	nd	nd	nd	nd
20:4n6	nd	nd	nd	nd	nd	0.56 ± 0.02	0.89 ± 0.02*	0.59 ± 0.00
∑n-6	0.99 ± 0.04*	0.36 ± 0.04	8.37 ± 1.24	12.8 ± 0.25*	12.4 ± 1.18*	9.85 ± 0.35	1.43 ± 0.02*	1.04 ± 0.02
18:3n3	0.72 ± 0.06*	0.14 ± 0.05	2.68 ± 0.45*	1.36 ± 0.11	10.2 ± 1.06	16.4 ± 0.54*	nd	nd
18:4n3	1.05 ± 0.08	1.55 ± 0.06*	11.2 ± 2.36*	4.84 ± 1.14	4.31 ± 0.49	10.2 ± 0.25*	nd	nd
20:5n3	6.22 ± 0.31*	4.86 ± 0.43	1.05 ± 0.19	1.68 ± 0.48	2.58 ± 0.24	5.05 ± 0.09*	2.59 ± 0.00	2.98 ± 0.07*
22:6n3	0.48 ± 0.04*	0.33 ± 0.00	10.3 ± 1.95	7.86 ± 1.03	nd	0.90 ± 0.05	nd	nd
∑n-3	8.36 ± 0.55*	6.64 ± 0.75	25.2 ± 4.82	14.9 ± 3.24*	17.6 ± 1.10	32.6 ± 0.91*	2.59 ± 0.00	2.98 ± 0.07*
∑PUFA	9.34 ± 0.57*	7.01 ± 0.76	33.6 ± 6.02	27.7 ± 3.21	30.1 ± 2.28	42.5 ± 1.25*	4.02 ± 0.02	4.02 ± 0.10

Fatty acid composition is expressed as percentage values of total fatty acids in TAG. Mean ± SE, n = 3. t-test were carried out for fatty acids in the same species. Asterisks indicate significant difference.

∑SFA, sum of saturated fatty acid; ∑MUFA, sum of mono unsaturated fatty acid; ∑PUFA, sum of polyunsaturated fatty acid; nd, not detected.

content of *N. oceanica* in our study was higher than previously found under N-repleted conditions by (Fakhry and El Maghraby, 2015). This suggests that *N. oceanica* accumulated lipid when growing under N-limitation. The lower lipid content under stronger N-limitation may be due to the improper harvesting time. Lipid per cell reached a maximum at day six and then started to decrease (Sandmann et al., 2018). This indicates that the harvesting of *N. oceanica* should be done at a time when lipid content is highest. *R. baltica* showed the greatest difference of lipid content between the two cultivation methods, with nearly a doubling in total lipid from moderate to strong N-limitation. This indicates that *R. baltica* responds strongest to stronger N-limitation among the four microalgae species, partially because of the phycobilin content (da Silva et al., 2009).

Next to content or yield of lipids, the localization of essential fatty acids is of great interest because of digestibility or processing challenges, and fatty acids are mainly localized in either storage lipids in neutral TAGs or in polar membranes. The fatty acid content of TAG increased dramatically under strong N-limitation in all species except for *N. oceanica*. This may be attributed to biosynthesis or conversion of existing polar membrane lipids into TAG (Xiao et al., 2013). The fatty acid content of PL decreased with stronger N-limitation in T-Iso and *N. oceanica*, whereas it increased in *R. baltica* and was the same in *P. tricornutum*. When algae are cultured for supply of PUFAs such as EPA and DHA in fish and animal feed, PL are nutritionally more available than those esterified into NL (Guedes et al., 2010). In our study, the percentage of EPA was found in a higher fraction in PL than in TAG in all species, and it decreased with stronger N-limitation. This indicates that EPA was mainly located in PL which decreased with strong N-limitation. T-Iso was found to have high fraction of DHA in PL but the percentage decreased in strong N-limitation. Therefore, microalgae should be cultured under N-repletion conditions to obtain higher EPA and DHA in PL when supplying for n-3 LC-PUFAs in fish feed.

In *P. tricornutum*, a higher MUFA content (dominated by 16:1n7), was found in strong N-limitation, due to TAG accumulation (Vu et al., 2015). The percentage of PUFA, including EPA which is among the most desired components for aquaculture, decreased almost 50% with stronger N-limitation, but the absolute content of EPA (mg g^{-1}) increased due to increased fatty acid content. This was also found in *P. cruentum* where N-limitation reduced the synthesis of n-3 PUFA while it induced accumulation of TAG (Breuer et al., 2012; Qiao et al., 2016).

Among the four species, T-Iso was the only species where PUFA content increased in strong N-limitation due to the increase in 18:4n-3 and 20:4n-6. We also found that T-Iso had the highest DHA content of the four species of microalgae (Harrison et al., 1990; Reitan et al., 1994; Breuer et al., 2012). The percentage of DHA in TAG increased by 30% with stronger N-limitation, which resulted in an increase of DHA in total lipid. Our study suggested that stronger N-limitation gave a desired increase in PUFA, especially the content of DHA in T-Iso.

TABLE 4 | Fatty acid profiles in polar lipid (PL) of four microalgae species cultured under nitrogen limitation, by batch (BT) and semi-continuous culture (SC).

% of total fatty acid	*P. tricornutum*		T-Iso		*R. baltica*		*N.oceanica*	
	BT	SC	BT	SC	BT	SC	BT	SC
14:0	2.72 ± 0.30	4.55 ± 0.25*	9.47 ± 0.29	13.0 ± 1.09*	4.83 ± 1.49	4.68 ± 1.53	3.98 ± 0.29	2.95 ± 0.23
16:0	20.3 ± 0.28	20.3 ± 1.25	11.3 ± 0.18	15.0 ± 2.45	16.1 ± 5.52	11.3 ± 2.12	23.4 ± 0.91	22.8 ± 2.43
18:0	2.87 ± 0.34*	1.51 ± 0.24	2.47 ± 0.23	1.84 ± 2.39	9.75 ± 6.84*	1.10 ± 0.63	6.73 ± 7.95	17.5 ± 5.42
20:0	nd	0.23 ± 0.02*	nd	nd	nd	nd	nd	nd
22:0	nd	0.67 ± 0.07*	nd	nd	nd	nd	nd	nd
∑SFA	25.8 ± 0.31	27.3 ± 1.48	23.3 ± 0.12	29.9 ± 3.57*	30.7 ± 12.5	17.1 ± 3.43	34.1 ± 8.58	43.4 ± 8.34
16:1n7	19.2 ± 0.17	21.6 ± 0.29*	1.80 ± 0.03	1.50 ± 0.19	2.09 ± 0.01*	0.99 ± 0.43	16.4 ± 2.03	11.0 ± 1.55
18:1n9	1.44 ± 0.07*	0.77 ± 0.04	17.8 ± 0.32*	15.6 ± 0.95	8.61 ± 5.59	2.13 ± 0.79	13.5 ± 1.69	7.99 ± 1.14
18:1n7	1.22 ± 0.01*	0.67 ± 0.02	1.09 ± 0.00	1.06 ± 0.10	1.55 ± 0.68	4.28 ± 0.31*	nd	nd
∑MUFA	22.2 ± 0.14	23.3 ± 0.29*	20.7 ± 0.35*	18.2 ± 0.85	12.3 ± 5.38	7.40 ± 1.50	29.9 ± 3.72	19.3 ± 2.26
18:2n6	2.60 ± 0.11*	2.01 ± 0.05	6.27 ± 0.12*	4.88 ± 0.29	5.29 ± 0.34	5.36 ± 0.39	2.24 ± 0.30	2.79 ± 0.43
20:4n6	1.04 ± 0.04*	0.15 ± 0.00	nd	nd	nd	0.94 ± 0.08	10.1 ± 1.41	7.45 ± 1.40
∑n-6	3.64 ± 0.15*	2.16 ± 0.05	6.27 ± 0.12*	4.93 ± 0.34	5.29 ± 0.34	6.30 ± 0.43*	12.4 ± 1.71	10.2 ± 1.84
18:3n3	1.03 ± 0.07*	0.22 ± 0.03	3.19 ± 0.06	3.45 ± 0.39	3.75 ± 0.80	16.0 ± 2.81*	nd	nd
18:4n3	1.53 ± 0.04	1.75 ± 0.05*	11.1 ± 0.27	10.6 ± 1.57	6.51 ± 1.69	17.9 ± 5.43*	nd	nd
20:5n3	22.0 ± 0.35	26.2 ± 0.36*	nd	nd	3.89 ± 2.75	12.1 ± 1.07*	21.1 ± 2.95	23.3 ± 4.21
22:6n3	3.65 ± 0.09	4.93 ± 0.09*	16.9 ± 0.47	19.1 ± 1.49	6.30 ± 3.79	12.8 ± 0.28*	nd	nd
∑n-3	28.2 ± 0.37	33.1 ± 0.40*	31.2 ± 0.79	33.3 ± 1.59	19.2 ± 3.73	58.8 ± 7.15*	21.1 ± 2.95	23.3 ± 4.21
∑PUFA	31.8 ± 0.53	35.2 ± 0.41*	37.5 ± 0.91	38.2 ± 1.81	24.4 ± 3.40	65.1 ± 7.20*	33.4 ± 4.65	33.5 ± 6.05

Fatty acid composition is expressed as percentage values of total fatty acids in PL. Mean ± SE, n = 3. t-test were carried out for fatty acids in the same species. Asterisks indicate significant difference.

∑SFA, sum of saturated fatty acid; ∑MUFA, sum of mono unsaturated fatty acid; ∑PUFA, sum of polyunsaturated fatty acid; nd, not detected.

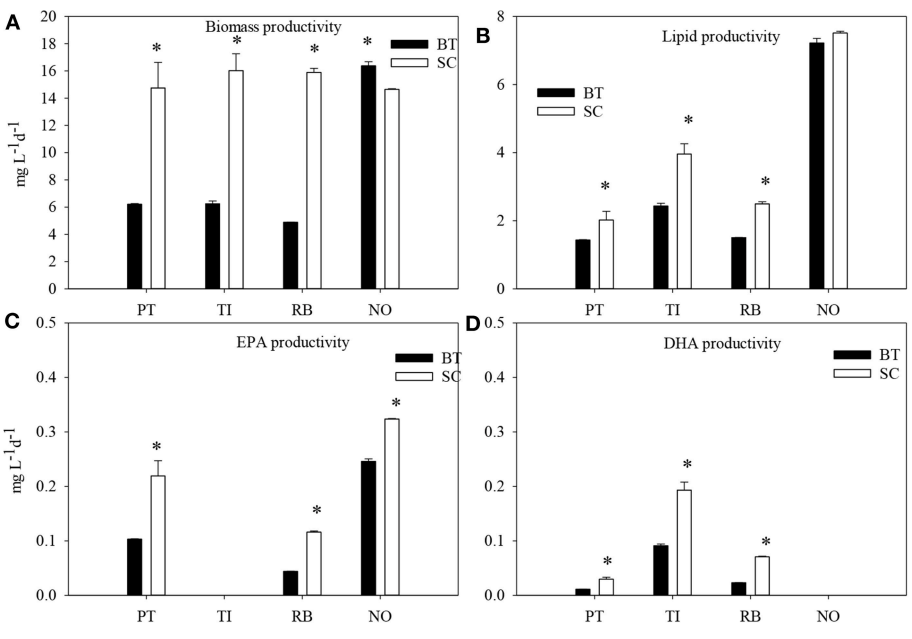

FIGURE 3 | The biomass productivity **(A)** lipid productivity **(B)**, EPA productivity **(C)** and DHA productivity **(D)** of four marine microalgae species cultured under nitrogen limitation, by batch (BT) and semi-continuous culture (SC). PT, *P. tricornutum*; TI, *Isochrysis aff. galbana* clone T-Iso; RB, *R. baltica*; NO, *N. oceanica.* Mean values with SE bars. *t*-test were carried out for productivity between BT and SC in the same species. Asterisks indicate significant difference.

In *R. baltica*, the percentage of MUFA increased with the increase N-limitation, and 18:1n9 was doubled: an effect that was found only in *R. baltic*a. The two fatty acids that dominate the PUFA content were 18:3n3 and 18:3n4, a pattern also found in previous studies of *Rhodomonas sp*. (Fernández-Reiriz et al., 1989; Patil et al., 2007). On the other hand, PUFA production

including EPA and DHA decreased with strong N-limitation. *R. baltica* was the only species that contained both EPA and DHA of the four studied species. The increase in PUFA in the algae diet would enhance the egg production and somatic growth of copepods (Rasdi and Qin, 2016) and the high EPA/DHA content explains why *R. baltica* especially is a preferred live feed for copepods in hatcheries (Zhang et al., 2013; Vu et al., 2015).

In *N. oceanica*, up to 86–87% of the fatty acids were in TAG, which is good for biodiesel production (Chen et al., 2015; Ma et al., 2016). The PUFA content, including EPA, was very low under N-limitation, and showed a slight decrease with stronger N-limitation. The nutrient limitation probably reduced the synthesis of PUFA (Sukenik, 1991; Reitan et al., 1994; Xiao et al., 2013).

Calculation of biomass productivity in batch systems depended on the size of the inoculum and the period until harvesting, and the comparison between different cultures is only a relative indicator. However, some general trends were observed, and the biomass productivity significantly decreased under strong N-limitation in all species except for *N. oceanica*. This in turn led to lower lipid productivity, although the strong N-limitation increased the lipid content more than 50%. The EPA and DHA productivities are both of great importance for exploitation of marine microalgae in aquaculture. Among the four species, *N. oceanica* showed the highest EPA productivity and it decreased with stronger N-limitation. The highest DHA productivity was found in T-Iso under moderate N-limitation. Based on the result, *N. oceanica* and T-Iso are two promising microalgae strains for producing an alternative source of n-3 LC-PUFAs under moderate N-limitation.

CONCLUSION

As shown in the present study, *N. oceanica* and T-Iso are two promising microalgae strains for producing an alternative source of n-3 LC-PUFAs under moderate N-limitation. N-limitation led to TAG accumulation, however, increased lipid content in microalgal cells under strong N-limitation may not increase the lipid productivity because biomass production usually is reduced. Therefore, a combination of approaches such as metabolic engineering, conditioning and selection may be required to further increase both lipid and n-3 LC-PUFA content without substantial loss of biomass.

AUTHOR CONTRIBUTIONS

XW and HF contributed to the running of the experiment, sample and data analysis. XW wrote the manuscript. KL analyzed lipid and fatty acid composition. MC participated in the experimental design and guided in sampling and data analysis and manuscript preparation. OV is the project leader of MIRA that funded the study and he contributed in the experimental design and manuscript writing. KR is the project leader of Micro-Feed which funded the study as well, he contributed in the experimental design and manuscript writing.

FUNDING

This study was part of two research projects Microbial raw materials as source for protein and EPA and DHA for use in aquafeed, No.248355 and Microbially produced raw materials for aquafeed, No.239001 which are, founded by ERANET-COFASP and Norwegian research council, respectively.

ACKNOWLEDGMENTS

We thank Åsmund Johansen and Torfinn Solvang from SINTEF Ocean for excellent technical assistance with the cultivation system and Marte Schei and Merethe Selnes for carbon and nitrogen analysis. We also thank the referees for their valuable comments.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.