Variation in the fatty acid profiles of two cold water diatoms grown under different temperature, light, and nutrient regimes

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Abstract
There is a growing demand for marine omega-3 fatty acids (FAs) that is produced in high amounts by some microalgae. Here we determined the FA profiles of two cold water adapted diatoms, Chaetoceros wighamii and Thalassiosira baltica. The cultures were acclimated to different temperatures (3, 7, 11, 15, and 19 °C) and irradiance (20, 40, 130, and 450 μmol photons m\textsuperscript{-2} s\textsuperscript{-1}) and the FA profiles were determined in exponential and stationary growth phases, the latter induced by different nutrient limitation (N, P, and Si). The maximum growth rate was obtained by both species at 11 °C, ≥ 130 μmol photons m\textsuperscript{-2} s\textsuperscript{-1} and was 0.8 day\textsuperscript{-1} and 0.6 day\textsuperscript{-1} for C. wighamii and T. baltica, respectively. Both species contained relatively high amounts of eicosapentaenoic acid (EPA). Thalassiosira baltica accumulated maximally ~ 30 mg EPA g\textsuperscript{-1} ash-free dry weight (AFDW) under Si-limitation. The content of docosahexaenoic acid (DHA) was lower, reaching up to 4 mg DHA g\textsuperscript{-1} AFDW in T. baltica. The concentration of EPA correlated positively with the chlorophyll a:carbon ratio, suggesting that it is bound to membranes in the photosynthetic apparatus and the EPA content in T. baltica was high enough to consider it as a potent candidate for cultivation under cold (< 15 °C) conditions. Covering a wide range of environmental conditions, the strongest differentiation in FA profiles was observed between the species with the growth phase/nutrient limitation pattern as the second most important driver of the FA composition.

Keywords Bacillariophyceae \& Microalgae \& Lipids \& Omega-3 \& 20:5n3 \& 22:6n3 \& Baltic sea

Introduction
Microalgae constitute an important source of fatty acids (FAs) in nature, and, with their high growth rate under optimal conditions, the cultivation of microalgae for commercial use has gained increasing interest over the past decades. There has been a lot of focus on developing biofuels from algal lipids (e.g., Williams and Laurens 2010), but also much higher value products can be extracted from microalgae. Omega-3 FAs have for example a significant market as dietary supplements valued at > 2 billion US$ (GVR 2020). Microalgae can produce relatively large amounts of omega-3 FAs, but the concentration depends on both the species and environmental conditions during growth (Guschina and Harwood 2009). There is a range of literature on different species and how growth limitations can increase the total FA content and specific FAs (e.g., Guschina and Harwood 2009; Jónasdóttir 2019).

Lipids are a diverse group of biomolecules built of FAs and have a range of intracellular functions in phytoplankton such as structural elements of cell membranes, as, for example, phospholipids and galactolipids, and as carbon storage forms such as in triacylglycerol (TAGs). The FAs are in turn made of hydrocarbon chains and can be further divided into saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) FAs depending on whether there are none, one, or two or more double bonds in the carbon chain, respectively. For a review of the lipid synthesis in algae, see, e.g., Khizin-Goldberg (2016).

The FA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the most conspicuous examples of marine PUFAs that are generally not produced by terrestrial plants, and both EPA and DHA have beneficial properties as part of a human diet (Swanson et al. 2012). Light and temperature affect the FA profile as, e.g., photosynthetic pigments, and the membranes surrounding them increase at low light...
(Falkowski and Raven 1997). In diatoms, these membranes seem to consist to a large extent of PUFAs, in particular EPA (Xu et al. 2010), in contrast to higher plants that to a larger extent have shorter chain PUFAs (18:3 and 16:3) in their plastid membranes (Douce and Joyard 1996). The light-dark cycle might also affect the FA profile, with continuous light providing more TAGs (Brown et al. 1996).

The FA synthesis in diatoms is rapidly up- or downregulated as a response to shifts in temperature and it relates to the regulation of the cellular metabolism (Liang et al. 2019). In addition, changes in temperature may affect the fluidity of the lipids bound to cellular membranes, and one of the ways to cope with decreasing temperatures is to increase the proportion of PUFAs that are more fluid than SFAs (Jiang and Gao 2004; Svenning et al. 2019). Although an increasing proportion of PUFAs with decreasing temperature seems to be a general phenomenon, there are large species-specific differences and this correlation does not necessarily exist in all species (Thompson et al. 1992).

During exponential growth algae channel their resources into growth, but when growth stops due to inorganic nutrient limitation, photosynthesis may still fix carbon that some species store in the form of lipids. Nitrogen limitation, in particular, seems to enhance storage lipid accumulation in TAGs and most of the FAs synthesized are generally SFAs (e.g., 16:0) and MUFAs (e.g., 16:1) (Guschina and Harwood 2009). Most of the work on algal FAs has been performed at temperatures > 10 °C and there is at present a gap in profiling FAs of cold water adapted phytoplankton (Artamonova et al. 2017). Considering algal cultivation in temperate areas, cultivating cold water species could be beneficial during winter time when the air temperature decreases < 10 °C. When the temperature decreases close to freezing, cold water adapted phytoplankton can still obtain high growth rates (Schwenk et al. 2013). Understanding how temperature affects the FA profiles would be important for species selection and optimizing the cultivation system.

Based on our previous work with cold water diatoms (Schwenk et al. 2013), we focus here on two species exhibiting high growth rates at low temperatures. Our main aim was to examine the variability in the FA profiles depending on temperature and light and comparing exponential growth with different inorganic nutrient (N, P, and Si) limitation regimes. We were particularly interested in the content of the long-chained PUFAs: EPA and DHA.

Materials and methods

Culture acclimation and growth

*Chaetoceros wighamii* and *Thalassiosira baltica* were sourced from the culture collection of the Tvärminne Zoological Station (strains TVCWI, TVTBA) and cultured in T2 medium at 6 PSU, which is a modified F/2 medium (Guillard 1975) with N:Si:P nutrient ratios adjusted to 16:8:1, previously suggested to be optimal for these diatoms (Spilling 2007). The cultures were grown in 2-L polycarbonate flasks (filled to 1.5 L) and acclimated to different temperature (3, 7, 11, 15, 19, and 23 °C) and irradiance (20, 40, 130, and 450 μmol photons m⁻² s⁻¹) from daylight fluorescent tubes, Philips TLD 965). The flasks were incubated in a temperature-regulated water bath and irradiance was adjusted with neutral density screens. During the temperature acclimation phase, we started at the lowest temperature, and during exponential growth, a subsample was inoculated into the following temperature. This was done in 4 °C increments until no growth was detected. The temperature range was different for the two species and the maximum temperature with growth was 11 °C and 19 °C for *C. wighamii* and *T. baltica*, respectively. The cultures were kept in suspension by bubbling with pre-filtered (0.2 μm) air. Cell growth was monitored daily by counting cells with a FlowCam (FluidImaging), which collects micrographs of individual cells passing through a flow cuvette.

On sampling days, a subsample was filtered for determining the dry weight (DW), ash-free dry weight (AFDW), particulate organic carbon (POC), and chlorophyll *a* (Chla). In addition, an aliquot was centrifuged (10,000 rpm), the pellet frozen, and stored at −80 °C prior to the determination of FAs.

For DW and AFDW, pre-rinsed and combusted 25-mm GF/F filters (Whatman) were used. The filters had been individually weighted before filtration and kept in plastic Petri dishes. A known volume (25–200 mL, depending on the biomass concentration) was filtered onto the filters and they were subsequently dried in an oven (60 °C) overnight (>8 h). The filters were weighed again to determine the DW. The filters were combusted at 450 °C (4 h) and the ash weight (AW) was determined by weighing the filters again. Finally, the AFDW was determined by subtracting the AW from the DW. For determining the POC, subsamples were filtered onto acid-washed and pre-combusted GF/F filters. These were dried and stored at room temperature before measurement of the POC with a mass spectrometer according to Koistinen et al. (2017). For Chla, we used GF/F filters and the Chla was extracted in 96% ethanol for 24 h at room temperature following the protocol of Jespersen and Christoffersen (1987). The Chla content was determined with a spectrophotometer (Cary Varian Eclipse) calibrated against pure Chla standards (Sigma) using 430-nm excitation and recording the 670-nm emission light.

The exponential growth sampling was carried out when the biomass was 10–50% of the maximum (in terms of POC). After the sampling, all but 100 mL of the culture was removed and a new medium with limiting N, P, or Si was added. This procedure was done for a subsample of initial treatments,
representing five combinations of temperature and light conditions (Supplementary Table S1). All but the limiting nutrient (kept at the original concentration) were added in 5-fold concentrations. Growth was monitored as described above until biomass did not increase over three consecutive days. The second set of measurements was taken during this early stationary growth phase.

**Determination of fatty acids**

We added 500 μL of chilled (−20 °C) methanol with 0.1% butylated hydroxytoluene to the 5-mg DW algae sample in order to break the cells. The resulting solution was incubated at −20 °C for 10 min in reaction vials. The algal samples were further disrupted with two 4-mm stainless steel balls in a mixer mill (Retsch MM 301) for 3 min at 25 Hz. Temperature for further disrupted with two 4-mm stainless steel balls in a mixer mill (Retsch MM 301) for 3 min at 25 Hz. Temperature for these steps was kept <0 °C by pre-chilling all equipment.

The following steps were performed at room temperature: 1000 μL chloroform and 150 μL internal standard (1549 mg L−1 triheptadecanoin, Sigma-Aldrich, 1029.6 mg L−3 heptadecanoic acid in chloroform:methanol, 2:1, Fluka, Sigma-Aldrich) was added to each sample and mixed for 10 min. After centrifugation, the supernatant was acidified with 300 μL, 20 mM acetic acid, mixed for 5 to 10 min, and centrifuged again. The organic phase was extracted twice using 500 μL chloroform, mixing for 10 min, and centrifugation. Organic phases were pooled and a subsample of 750 μL was dried in a glass tube under nitrogen flow. The residue was dissolved in 300 μL isopropanol. Each sample was extracted and the FAs were determined in duplicates. The average of these measurements was used for the data analysis.

The FAs were determined using transmethylation and gas chromatography (GC) with a flame ionization detector (FID). The samples were dried again under nitrogen flow, taken up in 700 μL petroleum-ether, and 125 μL sodium methoxide (97%, dissolved in methanol) was added before boiling the solution at 45 °C for 5 min. After cooling, 500 μL NaHSO4 (15% m/v) and 200 μL petroleum-ether were added and the samples were mixed. After separation of the two phases by centrifugation, the petroleum-ether phase was transferred to a GC vial. The solvent was evaporated and the residue was dissolved in 1000 μL hexane. Subsequently, 1 μL of this solution was used for further gas chromatography (GC) analysis. This transmethylation procedure does not methylate the free fatty acids (FFA), allowing distinction of FFA and structural FAs in the following GC step.

FAs were separated and evaluated quantitatively by using a capillary GC (7890A with sampler CTC ANALYTICS GC-PAL SYSTEM, Agilent Technologies) equipped with a BP-21 column (25 m × 0.2 mm × 0.3 mm, HP-FFAP Polyethylene Glycol TP, Agilent) and connected to a FID. The injector temperature was 260 °C. For every sample, 1 μL was injected and transferred splitless to the column. The oven temperature was programmed to increase from 70 (1.5 min) to 240 °C at the rate of 7 °C min−1. The carrier gas was helium with pressure of 16.671 psi. Peaks were allocated to substances via multicapillary column gas chromatography and via comparison to reference substances (F.A.M.E. Mix, #1891, Supelco, Sigma-Aldrich). Altogether, 30 FAs or FA methyl esters were identified and quantified.

**Data treatment**

Statistical analysis was carried out using SigmaPlot 13. Student’s t test was used to test for differences between two groups. For comparing more groups, we used analysis of variance (ANOVA) followed by a post hoc test: Tukey, when the variance between groups was similar, or on ranks followed by Dunn’s method if the variances between groups were different.

An ordination plot (non-metric multidimensional scaling, NMDS) was used to identify links between the species, growth conditions, and FA profile. The coordinates of each sampling along the NMDS axes 1 and 2 were used as explanatory variables in separate generalized additive models (GAMs), using R packages “vegan” (metaMDS function) and “mgcv” (Wood 2016). The response variables were used to color the symbols in the resulting NMDS plots, using R packages “colorRamps” (Keitt 2008) and “plotrix” (Lemon 2006).

**Results**

For both species, the maximum growth rate was obtained at 11 °C and an irradiance of ≥130 μmol photons m−2 s−1, but the species differed in growth rate with increasing temperature (Fig. 1). *Chaetoceros wighamii* had the highest maximum growth rate at ~0.8 day−1, and 11 °C was the maximum temperature where it grew. *Thalassiosira baltica* had a slightly lower maximum growth rate at ~0.6 day−1 and grew at lower rates up to 19 °C.

During exponential growth, the harvested biomass was similar for both species at roughly 50 mg AFDW L−1 (Fig. 2). The biomass concentration increased in the stationary growth phase and with the growth medium used, which was adjusted to prevent too high biomass and self-shading (light) effects. The highest biomass harvest was in the N- and P-limited cultures with roughly 250–350 mg AFDW L−1. The harvested AFDW was lower during Si-limitation, in particular for *T. baltica* (~100 mg AFDW L−1; Fig. 2).

The share of FAs of the biomass was affected by the growth phase, and there was a clear difference between the species (Fig. 2). *Thalassiosira baltica* had a higher FA content during exponential growth at 7% of AFDW compared with *C. wighamii* at 3% of AFDW (t test, p = 0.006). For both species, the FA content increased in the stationary growth
phase, but for *T. baltica*, Si-limitation produced the highest average FA content, which was higher than exponential growth and N-limitation (Dunn, \( p < 0.04 \)) but was statistically not different from P-limitation (Dunn, \( p = 0.3 \)). For *C. wighamii*, N-limitation increased the FA content most (Tukey, \( p < 0.001 \)) to an average of 46% of AFDW (Fig. 2).

In ordination, the strongest differentiation in FA profiles was observed between the species (\( R^2 = 0.77 \) of MDS1, \( p < 0.0001 \); Fig. 3), with the growth phase/limitation pattern as the second most important driver of the sample FA composition (Supplementary Fig S1). The main difference between the species was the much higher proportion of free FAs in *C. wighamii* while *T. baltica* contained more of the FAs: 18:4, 20:3, and 22:5 (Fig. 3, Table S1).

Although the species and growth phase were the dominating factors influencing the full FA profile, there were also effects of light and temperature in individual FAs. During exponential growth, the share of PUFAs was clearly highest in *T. baltica* (Fig S2). For this species, there was both a temperature and light effect with a higher proportion of PUFA at low temperature and low light at close to 50% of the total FAs. In *C. wighamii*, the overall PUFA content was < 30% of total FAs during exponential growth and it was highest at low light with no apparent temperature effect (Fig S2, Table S1).

The share of MUFA was higher in the stationary growth phase compared with nutrient replete growth for both species (\( p \leq 0.02 \)) apart from exponential growth and N-limitation in *T. baltica* (Fig S2, Dunn, \( p = 0.2 \)). Most of this increased share in MUFA was the FA 16:1 (Table S1). The share of PUFA was highest in the exponential growth in *T. baltica* compared with all nutrient-limited stationary growth phases (Fig S2, Tukey, \( p < 0.02 \)). In *C. wighamii*, only a difference between exponential and N- and P-limited growth in PUFAs was observed (Dunn, \( p \leq 0.01 \)).

The highest eicosapentaenoic acid (EPA) content was found in *T. baltica* (Fig. 4). Out of the total FAs, the highest share of EPA was obtained during exponential growth, but the highest content of EPA in the AFDW was found during Si-limitation and the highest overall concentration during N-limitation (Fig. 4). For *C. wighamii*, the highest content of EPA was found during N-limitation both in relation to AFDW biomass and concentration per volume (Fig. 4). The concentration of docosahexaenoic acid (DHA) was lower in both species being < 20% of the EPA concentration under most conditions, except for *C. wighamii* during exponential growth where it was a bit higher (up to 36% of the EPA content). The DHA content was highest in *T. baltica* with up to 4 mg DHA g\(^{-1}\) AFDW.

There was a clear positive correlation between the EPA share of the total FAs and the Chl:a:C ratio (Fig. 5). This was most pronounced for *T. baltica* that had a steeper slope and higher coefficient of determination \( (R^2) \) compared with *C. wighamii*. It was also more pronounced in the exponential growth phase as the Chl:a:C ratio was in the low end during the stationary growth phase with a few exceptions during Si-limitation in *C. wighamii*. 

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**Fig. 1** The growth rate (day\(^{-1}\)) of *Chaetoceros wighamii* and *Thalassiosira baltica* grown under nutrient replete conditions in a different light (16:8 light-dark cycle) and temperature. The white dots represent the sampling points. *C. wighamii* and *T. baltica* did not grow at 15 and 23 °C, respectively. The growth rate of *C. wighamii* was redrawn from Spilling et al. (2015)
Discussion

The two diatoms cultivated here are naturally occurring in the Baltic Sea during winter and spring and represent two of the most dominating diatoms during this time period. *T. baltica* is larger in size than *C. wighamii* but occupies a similar niche in the ecosystem (Spilling 2007). There are some distinctions; however, *T. baltica* has a lower maximum growth rate as revealed in this study and apparently also allocates its carbon differently, e.g., provides less exudates for heterotrophic bacteria in a natural environment (Camarena-Gómez et al. 2021). It is interesting that the FA profiles differ so much implying relevant differences in food quality for higher trophic levels, i.e., a different potential for feed cultivation of these species. From a perspective of extracting omega-3 FAs, *T. baltica* is the preferred candidate with a very high proportion of EPA. *Chaetoceros wighamii* would be the better species if SFA is the preferred product, which could be the case for, e.g., paraffinic biodiesel made from hydrotreated vegetable oils.

In the literature there are many ways to present FA data. Most often, individual FAs are presented as a percentage of total FAs or alternatively as a share of a biomass measure such as DW, AFDW, or carbon content. For the latter, some form of DW is often reported and to compare with other studies we related the EPA content primarily to the AFDW. Diatoms exhibit a higher ash content than other microalgal groups due to their silica frustule (Nalewajko 1966), and for this reason, we used AFDW rather than DW. Using DW creates a bias when comparing diatoms with other algal groups and when comparing different diatoms because some species are more silicified than others. Even within the same species, the degree of silicification may differ, e.g., due to Si-limitation, which may shift the FA content per DW unit when the ash content decreases (Steinrücken et al. 2018).

EPA clearly constituted the most prominent PUFA in both the species, and its concentration as a percentage of total FAs was clearly higher at low light acclimation. The positive correlation with the Chlα:C ratio indicates that it
is bound to photosynthetic pigments. These contain a lot of membranes and it is likely that the main proportion of EPA was plastid membranes, which was also concluded by Thompson et al. (1992). Xu et al. (2010) identified EPA in photosynthetic glycerolipids in the diatom Stephanodiscus sp. Interestingly, the positive correlation between EPA and the Chlα:C ratio differed between the two species suggesting a difference in EPA content in these membranes. Although low light produced the highest share of EPA of total FAs, it is worth to note that the absolute concentration of EPA did correlate with light, as the total biomass/FA content in higher light compensated for the lower percentage EPA of the total FAs.

Low temperature influenced the FA content in *T. baltica* with higher PUFAs (including EPA) when the temperature decreased to 3 °C. This was not the case in *C. wighamii* supporting that temperature effects on PUFAs are species specific (Thompson et al. 1992).

Considering commercial cultivation of these species, a central question is how well the EPA content of *T. baltica* compares to other alternative species. In the review by Jónasdóttir (2019), diatoms were pointed out as one of the best groups containing highly unsaturated FAs (HUFA) including EPA and DHA at 2.3 ± 3.1% (SD) of total carbon content. According to our results, *T. baltica* contained up to 7.7 ± 1.0% (SD) HUFA of the POC content under Si-limitation. The total biomass produced under these conditions was low, however, but the HUFA content was still 4.0 ± 0.3% (SD) of POC on average in the N-limited biomass, which is still high compared with the numbers presented in Jónasdóttir (2019). The amount of EPA at 3% of AFDW was comparable to the best values obtained with *P. tricornutum* (Jiang and Gao 2004). *Thalassiosira baltica* had a higher share of EPA than comparable cold water diatoms (Artamonova et al. 2017; Steinrücken et al. 2017, 2018; Peltomaa et al. 2019), with the exception of *Porosira glacialis* that provides up to 10%
EPA of total FAs (Svenning et al. 2019). *Thalassiosira baltica* could thus be a good candidate species for EPA production in a brackish, cold water cultivation system. However, Peltomaa et al. (2019) included a strain of *T. baltica* in their screening for FAs that had a much lower FA share overall, including EPA, and the suitability needs to be checked in individual strains.

In summary, the two cold water diatoms *C. wighamii* and *T. baltica* had very different FA profiles and the content of individual FAs was differentially affected by temperature, light, and nutrient limitation. Both species contained EPA, whereas the concentration of DHA was much lower in both species. Especially, *T. baltica* reached a high concentration of EPA up to levels comparable to the highest EPA producers found in the literature. The EPA content as a percentage of total FAs was positively correlated with the Chl:a:C ratio, suggesting that most of it is bound to membranes of the photosynthetic apparatus. However, higher biomass at high light compensated for a lower fraction of EPA and the total EPA content was not affected by the light level. Covering a wide range of environmental conditions, the strongest differentiation in FA profiles was observed between the species with the growth phase/nutrient limitation pattern as the second most important driver of the FA composition.

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Authors' contributions K.S., J.S., and T.T. organized and carried out the experiment. D.S. and H.R. carried out the measurements of fatty acids. K.S. wrote the manuscript with input from all other co-authors.

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Data Availability All data is included as supplementary material.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Consent for publication All coauthors consent to the present version of the manuscript for submission for publication.

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