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## Combined effects of eutrophication and warming on polyunsaturated fatty acids in complex phytoplankton communities: A mesocosm experiment



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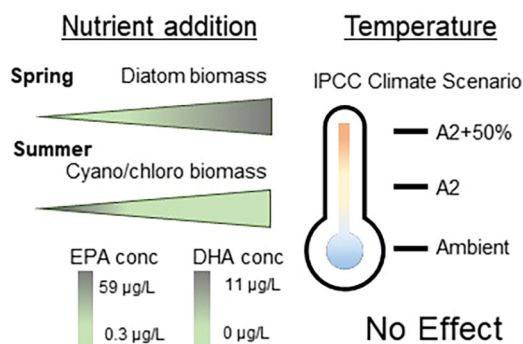
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### HIGHLIGHTS

- We analyzed the impact of warming and nutrient additions on the concentration of EPA and DHA in complex phytoplankton communities.
- The concentrations of EPA and DHA were largely driven by phytoplankton community composition and biomass.
- Nutrient addition increased phytoplankton biomass and thus the concentrations of EPA and DHA in spring.
- Warming had marginal effects on seston fatty acid profiles or the concentration of EPA and DHA.
- N:P ratio and warming had an interactive effect on phytoplankton EPA and DHA concentrations.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Climate change and eutrophication are among the main stressors of shallow freshwater ecosystems, and their effects on phytoplankton community structure and primary production have been studied extensively. However, their combined effects on the algal production of polyunsaturated fatty acids (PUFA), specifically, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are currently unresolved. Moreover, the proximate reasons for changes in phytoplankton EPA and DHA concentrations are unclear, i.e., the relative importance of ecological (changes in the community composition) vs. ecophysiological (within taxa changes in EPA and DHA levels) factors.

We investigated the responses of phytoplankton EPA and DHA concentrations to warming (IPCC climate scenario) and nutrient additions in mesocosms which had been run continuously at varying temperature and nutrient levels for 15 years prior to this study. Nutrient treatment had a significant effect on phytoplankton EPA and DHA concentrations and about 59 % of the variation in EPA and DHA concentrations could be explained by changes in the phytoplankton community structure. Increased biomass of diatoms corresponded with high EPA and DHA concentrations, while cyanobacteria/chlorophyte dominated mesocosm had low EPA and DHA concentrations. Warming had only a

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marginal effect on the EPA and DHA concentrations in these mesocosms. However, a significant interaction was observed with warming and N:P ratio.

Our findings indicate that direct nutrient/temperature effects on algal physiology and PUFA metabolism were negligible and the changes in EPA and DHA concentrations were mostly related to the phytoplankton community structure and biomass. These results also imply that in shallow temperate lakes eutrophication, leading to increased dominance of cyanobacteria, will probably be a greater threat to phytoplankton EPA and DHA production than warming. EPA and DHA are nutritionally important for upper trophic level consumers and decreased production may impair secondary production.

## 1. Introduction

Climate change is predicted to increase the global mean temperature by 2–4 °C within the next century (IPCC, 2014). Shallow freshwater ecosystems are particularly susceptible to climate change because lake water temperature, including thermal stratification and periodic heat waves, chemistry, and hydrology are climate-dependent, and lakes and rivers are already exposed to numerous anthropogenic stressors, such as increased nutrient loading and subsequent eutrophication (Adrian et al., 2009; Woodward et al., 2010; Mooij et al., 2005; Kundzewicz et al., 2008; Jeppesen et al., 2009, 2011, 2021; Woolway et al., 2021). Therefore, multistressor studies are essential to fully understand the effects of climate change on aquatic ecosystems (Birk et al., 2020). Decreased algal production and/or trophic transfer of n-3 and n-6 polyunsaturated fatty acids (n-3 and n-6 PUFA) may be potential mechanisms altering ecosystem functioning because the availability of certain PUFA have been suggested to drive secondary production in lakes (Müller-Navarra et al., 2000). Specifically, eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic acids (22:6n-3, DHA) are considered important for maintaining normal growth and reproduction in most animals, especially aquatic organisms. EPA and DHA are mainly produced by algae and subsequently transferred through the food web to fish and top consumers via zooplankton and benthic macroinvertebrates. Increased loading of nutrients and dissolved organic carbon from the watershed have been linked with decreased mass fractions of EPA and DHA in game fish (Strandberg et al., 2016; Taipale et al., 2016). EPA and DHA have significant health benefits also for humans (Harris et al., 2021), and decreased mass fractions in fish may affect EPA and DHA intake and nutritional quality for humans (Strandberg et al., 2018).

Eutrophication and climate warming may affect phytoplankton production and EPA and DHA concentrations directly by altering the fatty acid profiles within specific phytoplankton taxa (Fernandes et al., 2016; Guchina and Harwood, 2006; Hazel, 1995; Hixson and Arts, 2016) or indirectly by altering phytoplankton community structure (Galloway and Winder, 2015; Taipale et al., 2016). The direct effects are usually investigated with algal monocultures (Fernandes et al., 2016; Hixson and Arts, 2016) while the indirect effects are inferred from field studies or simulations (Taipale et al., 2016; Strandberg et al., 2020). The fatty acid compositions of algae and cyanobacteria are strongly driven by phylogeny (Taipale et al., 2013; Galloway and Winder, 2015), albeit with some environmental influence, and these taxonomic differences are so distinct that seston fatty acid composition can be used to infer phytoplankton community structure (Strandberg et al., 2015; Cañavate, 2019). Eutrophication often increases the frequency, intensity and duration of cyanobacterial blooms (Carey et al., 2012), and since cyanobacteria do not synthesize long chain PUFAs this will decrease the production of EPA and DHA within phytoplankton communities (Strandberg et al., 2015; Taipale et al., 2016). Stronger and longer periods of thermal stratification and warming have also been noted to favour cyanobacteria (Carey et al., 2012; Woolway et al., 2021). Although phytoplankton fatty acid profiles are largely phylogenetically controlled, laboratory experiments on monocultures have shown that temperature and nutrient availability can directly alter fatty acid profiles in algae and cyanobacteria (Cañavate et al., 2017a; Fernandes et al., 2016; Hixson and Arts, 2016; Juneja et al., 2013; Los et al., 1997; Wada and Murata, 1990; Yaakob et al., 2021). For instance, increasing temperature

has been noted to decrease the proportion of n-3 PUFA and increase the proportion of n-6 PUFA in algae and cyanobacteria, leading to an overall decrease in the n-3/n-6 ratio. However, the variation in n-3 and n-6 proportions and their ratios are high and not all taxa followed this trend (de la Rosa et al., 2020; Renaud et al., 2002). Nutrient limitation generally leads to increased accumulation of triacylglycerol, which results in a higher content of saturated and monounsaturated fatty acids, SFA and MUFA, respectively (Juneja et al., 2013; Yaakob et al., 2021). However, the proportion of PUFA, specifically EPA and DHA, in algae have been noted to decrease with nutrient limitation (Fernandes et al., 2016).

The relative importance of direct ecophysiological vs. indirect population level responses to increasing nutrient loading and warming, and their possible interactions on the concentrations of EPA and DHA in complex phytoplankton communities is currently unresolved. However, an experiment with five phytoplankton taxa suggested that phosphorus availability and light condition alter fatty acid profiles in phytoplankton due to changes in the abundance as well as ecophysiological adaptation (Marzetz and Wacker, 2021). Improved knowledge of the role of the mechanisms by which environmental drivers alter the algal production of EPA and DHA is essential for understanding the effects on whole food webs, as well as for lake and fisheries management and restoration purposes. The main goal of the current study was to evaluate the proximate reasons for the changes in the EPA and DHA concentrations in phytoplankton community in response to increased nutrient loading and warming, based on mesocosm experiments, which enabled manipulation of environmental drivers and detect responses in complex phytoplankton communities. We hypothesize that the proximate reasons for changes in EPA and DHA concentration to the treatments are changes in the phytoplankton community structure and biomass (Strandberg et al., 2020; Wauthy and Rautio, 2020), and direct ecophysiological changes in phytoplankton will have only a marginal effect on the EPA and DHA concentration. We further hypothesize that nutrient addition and season have an interactive effect on the concentrations of EPA and DHA in phytoplankton. Large seasonal changes in environmental factors, nutrient availability and selective grazing pressure periodically shift phytoplankton community structure (Tilman et al., 1982; Bergquist et al., 1985). As a result, this seasonal succession will be an important predictor for phytoplankton EPA and DHA, specifically in high nutrient conditions. Finally, we hypothesized that temperature is not a major driver for the concentrations of EPA and DHA in phytoplankton in any nutrient treatments, because previous mesocosm studies have shown that nutrient loading drives phytoplankton biomass and community structure to a larger extent than temperature in experimental systems (Moss et al., 2003; Özen et al., 2013).

## 2. Material and methods

We experimentally manipulated water temperature (climate scenario) and the concentrations of nitrogen (N) and phosphorus (P) (nutrient manipulation) in outdoor mesocosms and investigated the responses of phytoplankton fatty acids, in particular EPA and DHA concentrations. The study was conducted in mesocosms located in Lemming, Central Jutland, Denmark (56°14'N, 9°31'E). These mesocosms were established in August 2003 and this is the longest continuously running lake mesocosm experiment in the world. Details of the mesocosm design can be found in

Liboriussen et al. (2005). Briefly, the setup consists of 24 fully mixed outdoor flow-through cylindrical stainless-steel tanks (diameter 1.9 m total depth 1.5 m) with semicontinuous addition of water (ESM Fig. S1). The flow-system enables a constant water level (1.0 m) and total volume (about 2800 L). The bottom of the mesocosms are covered with a 0.1 m layer of washed sand and on top of that a 0.1 m layer of sediment. Mesocosms are heated with three electrically powered (230 V AC) stainless steel heating elements (750 W each). Unheated mesocosms are equipped with 'dummy heating elements'. Mesocosms are constantly mixed with a paddle-shaped mixer without visibly disturbing the sediment. Mesocosms contain also periphyton and submerged macrophytes, mainly *Elodea canadensis* and *Potamogeton crispus* (Pacheco et al., 2021), as well as small planktivorous fish (*Gasterosteus aculeatus*). All mesocosms received nutrients from the groundwater input in which total P (TP) and total N (TN) concentrations have varied with time, in 2014 averaging 0.01 (range 0–2)  $\mu\text{g TP/L}$ , and 3.2 (range 2–3.8)  $\text{mg TN/L}$ , equivalent to 0.15  $\text{mg P/m}^2/\text{d}$  and 43  $\text{mg N/m}^2/\text{d}$  on average. Half of the 24 mesocosms had high nutrient treatment with an additional weekly loading of 2.7  $\text{mg P/m}^2/\text{d}$  ( $\text{Na}_2\text{HPO}_4$ ) and 27  $\text{mg N/m}^2/\text{d}$  ( $\text{Ca}(\text{NO}_3)_2$ ), giving a N:P ratio of 10 slightly above the Redfield ratio leaving room for expected loss of N by denitrification. The experiment was conducted as part of larger experiment involving EU-funded Transnational Access to European Aquatic Mesocosm Facilities (AQUACOSM) network. The experimental design consisted of temperature, i.e., climate scenarios, and nutrient manipulations (ESM Fig. S1). The climate scenarios included three treatments: 1) ambient (unheated reference), 2) IPCC A2 and 2) IPCC A2 + 50 % simulations (Houghton et al., 2001). The climate scenario A2 correspond to 2–3 °C increase and the A2 + 50 % 5–6 °C above ambient water temperature (Houghton et al., 2001). Nutrient manipulations included three treatments: 1) control, which received only groundwater, i.e., no additional nutrient additions, and represented low nutrient conditions, and two treatments with additional nutrient additions, representing high nutrient conditions: 2) P addition and 3) addition of both P and N. Nutrient input and variation in the N:P ratio control phytoplankton production and community structure (Abell et al., 2010). On June 14, 2018, the N additions were discontinued to decrease the N:P ratio potentially inducing N limitation (Pacheco et al., 2021). The N additions resumed on June 14, 2019. Nutrients (total phosphorus, ortho-phosphorus ( $\text{PO}_4\text{-P}$ ), total nitrogen,  $\text{NH}_4$  and  $\text{NO}_3\text{-N}$ ), chlorophyll *a*, conductivity and turbidity were determined weekly. Total N was analyzed according to Solorzano and Sharp (1980),  $\text{PO}_4\text{-P}$  according to Grasshoff et al. (1983) and nitrate + nitrite using a cadmium reduction method (Grasshoff et al., 1983). Chlorophyll *a* was extracted with ethanol and determined spectrophotometrically (Jespersen and Christoffersen, 1987). Turbidity and conductivity were weekly measured with an YSI650 MDS multiprobe and pH with a probe (OxyGuard®), light-duty submersion type connected to a Manta pH measurement system).

We sampled seston on May 24, 2018, and May 21, 2019, i.e., before and after the manipulation of the N:P loading ratio. We sampled seston also on July 31, 2019, i.e., 47 days after resuming nitrogen additions, to evaluate possible seasonal differences in the EPA and DHA concentrations. Unfortunately, we do not have summer samples representing systems with a low N:P loading ratio. The climate scenarios (ambient, A2 and A2 + 50 %) are the same for all sampling occasions. We collected water with a 1-m-long tube sampler while avoiding plants and cross-contamination with epiphytes. The water was pre-screened with a 50  $\mu\text{m}$  sieve to exclude zooplankton. The pre-screened water was filtered on a Durapore® filter with a pore size of 0.45  $\mu\text{m}$ . The filtered volume varied from 20 mL to 1200 mL depending on the seston biomass. The filters were immediately placed in methanol, flushed with  $\text{N}_2$ , closed with Teflon lined caps, and stored in  $-20$  °C.

The samples from 2018 were cryo-shipped to University of Eastern Finland (UEF) and the fatty acids were extracted and transmethylated within a month of collection. The 2019 samples were processed at the University of Aarhus, Silkeborg, within a week from sampling and cryo-shipped to UEF as fatty acid methyl esters. The free fatty acid 23:0 was used as an internal standard. The details of fatty acid extraction and transmethylation methods can be found in Strandberg et al. (2020). Briefly,

we used Folch extraction (Folch et al., 1957) followed by an acid catalyzed transmethylation reaction. The samples were analyzed at UEF with 6890 N gas chromatograph (Agilent Technologies) equipped with 5973 N mass selective detector (Agilent Technologies). We used a DB-23 column (length 60 m, diameter 0.25 mm, film thickness 0.15  $\mu\text{m}$ ) with helium as a carrier gas (average velocity 26 cm/sec). We used splitless injection and the temperature method was as follows: initial column temperature of 50 °C was maintained for 1 min after which the temperature was raised by 15 °C/min until reaching 150 °C, then by 0.5 °C/min until reaching 170 °C and finally by 2 °C  $\text{min}^{-1}$  until 230 °C. Peaks were identified based on mass spectra and fatty acid methyl ester standard mix GLC-538 (Nu-chek prep.), which was also used for calibration. Results are expressed as  $\mu\text{g FA/L}$  or as weight percentage (w%).

### 2.1. Taxonomic biomarkers

We used taxa-specific fatty acid biomarkers to infer the dominant phytoplankton groups in the mesocosms (Strandberg et al., 2015; Cañavate et al., 2017b; Cañavate, 2019). Although, environmental conditions are known to affect the fatty acid profiles in phytoplankton, the phylogenetic differences are significantly more important predictors for the algal and cyanobacterial fatty acid profiles (Galloway and Winder, 2015). Thus, the fatty acid biomarker approach can be used to infer the phytoplankton community structure at a group level, regardless of the environmental conditions (Pond et al., 1998; Dijkman and Kromkamp, 2006; Strandberg et al., 2015; Cañavate et al., 2017b; Cañavate, 2019). Biomarkers indicating cyanobacteria/chlorophyte dominance in seston include 16:2n-6, 16:3n-3, 16:3n-6, 16:4n-3, 18:2n-6 and 18:3n-6 (Taipale et al., 2013; Strandberg et al., 2015; Cañavate, 2019). Although, we cannot differentiate cyanobacteria from chlorophyte with the fatty acid biomarker approach, neither of these groups contain EPA or DHA. Thus, for the purpose of this study, the fatty acid biomarker approach provides adequate resolution. The  $\text{C}_{18}$  PUFA 18:3n-3 and 18:4n-3 are very abundant in chlorophytes, but because these fatty acids are found in many different algae, such as various photosynthetic flagellates, we chose to exclude them as taxonomic markers in this study. We used 18:5n-3 and 22:5n-6 as biomarkers for flagellates, such as cryptophytes, chrysophytes and dinoflagellates; 20:5n-3, and 22:6n-3 are also abundant in flagellates but since we aim to investigate the potential of the taxa-specific fatty acid markers to explain seston concentrations of 20:5n-3 and 22:6n-3, these variables were not used as biomarkers. Biomarkers indicating diatom dominance include the following highly specific fatty acids: 16:2n-4, 16:2n-7, 16:4n-1, 16:3n-4 and 18:4n-4. Additionally, 14:0, 16:1n-7 and 20:5n-3 are typically abundant in most diatoms, but as mentioned above 20:5n-3 was not used as a marker fatty acid. Also, 16:1n-7 is common and abundant in many organisms, including e.g., certain bacteria, and 14:0 is found widely in phytoplankton, including cyanobacteria, thus these fatty acids were excluded as taxa-specific biomarkers in this study (Taipale et al., 2013; Strandberg et al., 2015; Cañavate, 2019).

### 2.2. Statistical analyses

We used permutational analysis of variance to test for differences between the treatments for EPA and DHA concentrations in phytoplankton. We conducted a three-way ANOVA using nutrient treatment, climate scenario and season as fixed factors. Nutrient treatment had three levels: 'Control', 'N + P', and 'P'; climate scenario had also three levels: 'Ambient', 'A2' and 'A2 + 50 %'; and season had two levels: 'May' and 'July'. Data were  $\log(x + 1)$  transformed prior to analyses. Euclidean distance was used as the resemblance matrix and permutation of residuals was conducted under reduced model with type III (partial) sum of squares. The number of permutations was 999. For the calculation of effect size (% explained) negative estimates of components of variation were treated as zero. We also used PERMANOVA to assess the differences in the fatty acid profiles (w%) and n-3/n-6 ratio in phytoplankton between the above-mentioned treatments

(nutrients, climate scenario and season). The proportional data was arcsine square root transformed prior to PERMANOVA analysis.

A distance based linear model (DistLM) was used to further investigate the explanatory power of selected environmental predictors on the concentration of EPA and DHA in the phytoplankton. Based on the multicollinearity of selected environmental predictors (see ESM Table S1 for correlation coefficients), the predictors were classified into the following indicators: ‘Biomass’ (turbidity and the concentrations of chl-a, total P, and total N), and ‘Water chemistry’ (conductivity and pH). Phytoplankton community composition was classified into the following taxonomic indicators: ‘Diatoms’, ‘Flagellates’ and ‘Cyanobacteria/Chlorophyte’ (see section “Taxonomic biomarkers” for details). We also used the measured mesocosm temperature as an independent continuous predictor and nutrient treatments (i.e., control, P, N + P) as a categorical predictor in the model. Although, based on the PERMANOVA analysis, the nutrient additions were found to be strongly linked with phytoplankton biomass, we added the nutrient additions as a categorical predictor to detect any residual variation, which was not captured by the ‘Biomass’ indicator. Continuous predictors were normalized prior to the DistLM analysis. All multivariate analyses were done with Primer 6.1.15 with PERMANOVA 1.0.5 add-on (Primer-E Ltd.). We also analyzed the correlation between EPA and DHA concentrations in phytoplankton across all samples using IBM SPSS statistics version 27.

### 3. Results

#### 3.1. Temperature, chlorophyll a, and nutrients

The mean concentrations of chl-a, total P, and total N, temperature, turbidity, conductivity, and pH in mesocosms with different nutrient enrichment and climate scenarios at different sampling seasons (May 2018 and 2019 and July 2019) are presented in Table 1. In general, in the nutrient enriched mesocosms the concentrations of chl-a, total P and N were significantly higher than in the control mesocosms, which correspond to low nutrient conditions. In May the mean ± SD of chl-a concentration, across all climate scenarios, was 9 ± 19 µg/L in control mesocosms, 105 ± 81 µg/L in mesocosms with N + P additions and 48 ± 39 µg/L in mesocosms with only P additions. In July the mean chl-a concentrations were 15 ± 34 µg/L in control mesocosms and 215 ± 218 µg/L in mesocosms with N + P addition. Note that we do not have data for P only treatments in July. Ambient water temperature was significantly higher in July than in May in all mesocosms (Table 1). The A2 climate

scenario increased the water temperature 2–3 °C above the ambient temperature and the A2 + 50 % increased the water temperature about 4 °C above ambient (Table 1).

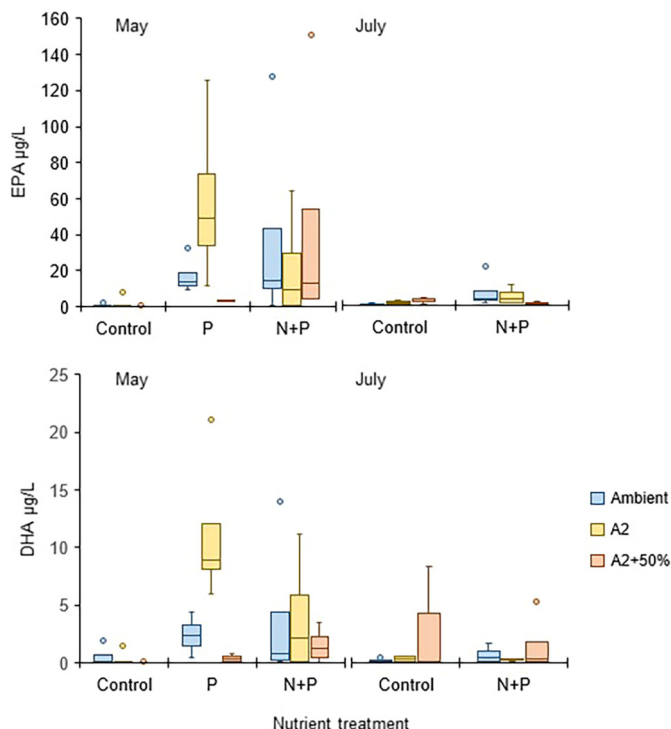
#### 3.2. EPA and DHA concentrations

The high nutrient mesocosms had significantly higher phytoplankton EPA and DHA concentrations than control mesocosms (low nutrient level) (Fig. 1, ESM Tables S1, S2 and S3). EPA concentrations were higher than DHA concentrations (Fig. 1), but the concentrations were highly correlated ( $r = 0.74$ , 95 % confidence interval 0.33–0.97,  $P < 0.001$ ). The low nutrient mesocosms always had low EPA and DHA concentrations. Also, the n-3/n-6 ratio was stable in the low nutrient mesocosms, regardless of the sampling season or climate scenario. Additionally, a significant interaction was observed for nutrients and the climate scenario and season (Table 2). Pairwise comparisons showed that the climate scenario did not affect the EPA concentrations in the control or in the N + P treatments, but in the P treatment the EPA concentration was significantly lower in the A2 + 50 % scenario than in the ambient or A2 scenarios (Table 3, Fig. 1). Variation in EPA concentrations in nutrient enriched mesocosms was high in spring, and on average the EPA concentrations were much higher in May than in July. Because of the high within group variation in the nutrient enriched mesocosms, we explored further which environmental factors, i.e., phytoplankton biomass, water chemistry, temperature, nutrient treatment or the abundance of diatoms, flagellates and cyanobacteria/chlorophytes best explained the variation in EPA and DHA concentrations. Distance based linear modeling indicated that the proportion of diatom biomarkers alone explained 59 % of the variability in phytoplankton EPA and DHA concentrations. The best model was achieved when algal biomass, flagellate biomarkers, and water chemistry indicators were also included as predictors (Table 4). Inclusion of cyanobacteria/chlorophyte biomarkers and nutrient treatment slightly improved the model, but this improvement was not statistically significant (Table 4). The model results are visualized in a dbrDA plot (Fig. 2). Measured temperature was not a significant predictor for phytoplankton EPA and DHA concentrations (Table 4). The DistLM analysis and the high explanatory power of phytoplankton taxonomic indicators and biomass imply that the effects of nutrient addition, climate scenario and sampling month on the concentrations of EPA and DHA were indirect, and the phytoplankton community composition was the proximate driver for EPA and DHA concentrations.

**Table 1**

Water chemistry parameters in the mesocosm with different nutrient (control, i.e., low nutrient condition, and P and N + P additions) and temperature treatments (Ambient and IPCC climate scenarios A2 and A2 + 50 %).

Month	Treatment		Temp.		Chl-a		TotP		TotN		Turbidity		Conductivity		pH	
	Climate	Nutrient	°C		µg/L		µg/L		µg/L		NTU		mS/m			
	Scenario	Addition	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
May 2018	Ambient	Control	18.9	0.8	40	29	29	10	578	196	13	21	0.37	0.06	8.1	0.6
	A2	Control	21.7	0.8	3	2	14	3	364	118	1	0	0.34	0.01	8.3	0.4
	A2 + 50 %	Control	22.9	0.8	3	2	15	7	352	71	1	1	0.34	0.03	8.6	0.6
	Ambient	N + P	19.6	0.4	149	102	261	218	2409	539	23	17	0.36	0.01	9.7	0.4
	A2	N + P	22.8	0.1	97	65	178	96	1686	513	23	24	0.38	0.03	9.5	0.7
	A2 + 50 %	N + P	23.8	0.5	70	11	197	35	1377	288	9	6	0.39	0.04	9.6	0.7
May 2019	Ambient	Control	19.3	0.4	2	1	13	5	434	85	2	2	0.32	0.02	8.7	1.1
	A2	Control	21.9	0.4	3	3	12	5	534	169	1	1	0.40	0.05	7.6	0.9
	A2 + 50 %	Control	23.2	0.4	2	1	9	5	433	142	1	1	0.35	0.04	7.5	0.6
	Ambient	P	19.6	0.2	61	50	154	59	855	300	9	8	0.36	0.02	9.6	0.3
	A2	P	22.3	0.1	64	30	200	89	1146	335	13	8	0.39	0.03	9.2	0.4
	A2 + 50 %	P	23.5	0.3	19	24	210	83	686	208	5	6	0.40	0.02	9.1	0.3
July 2019	Ambient	Control	22.6	0.3	4	4	16	4	213	45	0	0	0.32	0.03	8.5	1.1
	A2	Control	24.5	1.4	7	6	19	9	467	268	1	1	0.41	0.07	7.6	0.6
	A2 + 50 %	Control	25.4	2.1	31	58	28	26	543	429	1	1	0.43	0.07	7.5	0.4
	Ambient	N + P	22.8	0.2	271	304	268	120	1889	841	27	35	0.35	0.02	9.4	0.3
	A2	N + P	25.6	0.2	252	216	293	187	1649	942	37	40	0.39	0.02	8.4	1.7
	A2 + 50 %	N + P	26.8	0.3	123	135	270	160	863	296	4	3	0.42	0.03	8.8	0.2



**Fig. 1.** Phytoplankton concentrations (µg/L) of A) EPA and B) DHA in mesocosms with different nutrient treatments: control, i.e., low nutrient condition, and P or N + P addition and climate scenarios (Ambient, A2 and A2 + 50 %). Panels refer to spring (May) and summer (July) sampling.

### 3.3. Fatty acid profiles and the n-3/n-6 ratio

Temperature treatment was not a significant source of variation for the entire seston fatty acid profiles (PERMANOVA Pseudo-F = 0.490,  $P = 0.943$ , ESM Table S5). Nutrient treatment and sampling month had a significant interaction effect on phytoplankton fatty acid profiles (ESM Table S5). Fatty acid profiles in the low nutrient (control) mesocosms differed from those in the nutrient enriched mesocosms both in May and in July (ESM Table S6). Phytoplankton fatty acid profiles were not significantly different between the N + P and P treatments (ESM Table S6). Fatty acid profiles differed between May and July in the controls (Table S7, pairwise test  $t = 1.96$ ,  $P = 0.009$ ), but not in the N + P addition mesocosms ( $t =$

**Table 2**

PERMANOVA summary table for EPA and DHA concentrations in phytoplankton. Significant interactions are bolded.

Variable	Source of variation	df	SS	MS	Pseudo-F	P(MC)	Unique perms	% explained
EPA	Nutrient	2	37.15	18.575	23.525	0.001	998	29.9
	Climate scenario	2	5.9072	2.9536	3.7407	0.039	998	4.6
	Season	1	2.6414	2.6414	3.3453	0.066	997	2.4
	<b>Nutrient × Climate Scenario</b>	4	<b>11.461</b>	<b>2.8653</b>	<b>3.6289</b>	<b>0.014</b>	<b>999</b>	<b>10.5</b>
	<sup>a</sup> <b>Nutrient × Season</b>	1	<b>9.0266</b>	<b>9.0266</b>	<b>11.432</b>	<b>0.001</b>	<b>998</b>	<b>21.0</b>
	Climate Scenario × Season	2	0.86696	0.43348	0.549	0.556	998	0.0
	<sup>a</sup> Nutrients × Climate Scenario × Season	2	2.9523	1.4762	1.8695	0.174	998	5.2
	Residuals	56	44.217	0.78958				
	Total	70	120.44					
	DHA	Nutrient	2	7.357	3.6785	10.35	0.001	999
Climate scenario		2	2.2999	1.15	3.2355	0.036	999	5.8
Season		1	0.24167	0.24167	0.67994	0.416	998	0.0
<b>Nutrient × Climate Scenario</b>		4	<b>5.7812</b>	<b>1.4453</b>	<b>4.0664</b>	<b>0.007</b>	<b>999</b>	<b>18.8</b>
<sup>a</sup> <b>Nutrient × Season</b>		1	<b>1.6708</b>	<b>1.6708</b>	<b>4.7009</b>	<b>0.042</b>	<b>997</b>	<b>11.4</b>
Climate Scenario × Season		2	1.3189	0.65945	1.8554	0.143	997	4.0
<sup>a</sup> Nutrients × Climate Scenario × Season		2	0.27849	0.13925	0.39177	0.709	999	0.0
Residuals		56	19.904	0.35543				
Total		70	42.492					

<sup>a</sup> Missing term: Nutrient treatment ‘P’ not available in July.

**Table 3**

Pairwise comparisons of phytoplankton EPA and DHA concentrations in different nutrient treatments between climate scenarios (Ambient, A2 and A2 + 50 %) and sampling month (May and July), significant differences are bolded. Note that P treatment was available only for May samples, thus the pairwise comparison between sampling months could not be tested.

Variable	Nutrient treatment	Climate scenario/month	Climate scenario/month	t	P(MC)
EPA	Control	Ambient	A2	0.4199	0.691
		Ambient	A2 + 50 %	0.82709	0.427
		A2	A2 + 50 %	0.26363	0.808
		May	July	1.9293	0.07
		July	July	1.9293	0.07
	P	Ambient	A2	1.8242	0.107
		<b>Ambient</b>	<b>A2 + 50 %</b>	<b>4.6044</b>	<b>0.004</b>
		<b>A2</b>	<b>A2 + 50 %</b>	<b>4.7378</b>	<b>0.004</b>
		Ambient	A2	0.38621	0.698
		Ambient	A2 + 50 %	0.39563	0.698
N + P	Ambient	A2 + 50 %	0.0223	0.991	
	<b>May</b>	<b>July</b>	<b>2.4278</b>	<b>0.032</b>	
	Ambient	A2	0.1583	0.875	
	Ambient	A2 + 50 %	0.80777	0.444	
	A2	A2 + 50 %	1.0286	0.334	
DHA	Control	Ambient	A2	1.3717	0.197
		<b>Ambient</b>	<b>A2</b>	<b>3.4438</b>	<b>0.011</b>
		<b>Ambient</b>	<b>A2 + 50 %</b>	<b>2.6087</b>	<b>0.048</b>
		<b>A2</b>	<b>A2 + 50 %</b>	<b>7.6743</b>	<b>0.001</b>
		Ambient	A2	0.12074	0.912
	N + P	Ambient	A2 + 50 %	0.04955	0.965
		A2	A2 + 50 %	0.08128	0.951
		May	July	1.4402	0.158
		July	July	1.4402	0.158
		May	July	1.4402	0.158

1.44,  $P = 0.089$ ), likely because of the high variation in the fatty acid profiles in spring. Fatty acid profiles indicated that in spring the mesocosms with N + P additions were dominated either by diatoms or by cyanobacteria/chlorophytes (Fig. 3). In summer, none of the N + P mesocosms had a high proportion of diatom biomarkers, and the fatty acid data indicated cyanobacteria/chlorophyte dominance (Fig. 3). This was also reflected in the n-3/n-6 ratio, which, contrary to the entire fatty acid profile, significantly decreased in the N + P mesocosms from May to July (Fig. 4, ESM Table S8 and S9). Climate scenarios did not affect the n-3/n-6 ratio in any of the nutrient treatments or seasons (ESM Table S8).

### 4. Discussion

The results showed that EPA and DHA concentrations were strongly coupled to the phytoplankton biomass and community structure, specifically the abundance of diatoms. Together these predictors explained 73 % of the variation in the seston EPA and DHA concentrations. Consequently,

**Table 4**

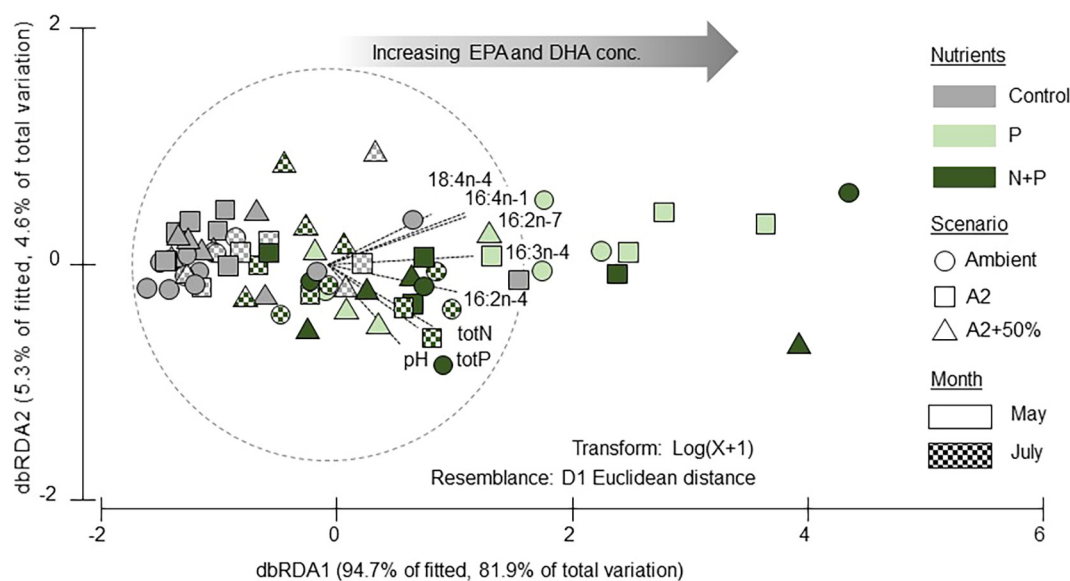
Marginal test results and the best model ( $Adj\ r^2 = 0.81$ ) from the DistLM analysis of phytoplankton EPA and DHA concentrations using the following indicators as independent predictors: biomass, water chemistry, temperature, as well as the proportions of specific phytoplankton biomarkers (diatoms, flagellates, and cyanobacteria/chlorophytes) and nutrient treatment. Variables that alone (i.e., ignoring other variables) explain a significant amount of the total variation in the data are bolded.

Marginal tests							
Indicator	SS (trace)	Pseudo-F	P	res. df	regr. df	Prop. explained	
Biomass	<b>44.679</b>	<b>6.234</b>	<b>0.002</b>	<b>66</b>	<b>5</b>	<b>0.27</b>	
Water chemistry	<b>50.604</b>	<b>15.317</b>	<b>0.001</b>	<b>68</b>	<b>3</b>	<b>0.31</b>	
Temperature	0.90578	0.38573	0.575	69	2	0.01	
Diatom biomarkers	<b>96.244</b>	<b>18.761</b>	<b>0.001</b>	<b>65</b>	<b>6</b>	<b>0.59</b>	
Cyano/chloro biomarkers	15.577	1.1276	0.332	64	7	0.10	
Flagellate biomarkers	3.2296	0.68758	0.529	68	3	0.02	
Nutrient treatment	<b>61.544</b>	<b>20.638</b>	<b>0.001</b>	<b>68</b>	<b>3</b>	<b>0.38</b>	
Step-wise tests							
Indicator	SS (trace)	Pseudo-F	P	res. df	regr. df	Prop. explained	Cumulative
Diatom biomarkers	96.244	18.761	0.001	65	6	0.59	0.59
+ Biomass	23.032	8.0456	0.001	61	10	0.14	0.73
+ Flagellate biomarkers	9.2274	7.9063	0.001	59	12	0.06	0.79
+ Water chemistry	5.1859	5.0541	0.006	57	14	0.03	0.82
+ Cyano/chloro biomarkers	5.3514	1.9039	0.069	51	20	0.03	0.85
+ Nutrient treatment	1.9268	2.1492	0.096	49	22	0.01	0.87

the direct temperature and nutrient effects on the seston EPA and DHA content were negligible. Most likely the 2–4 °C temperature increase was within the thermal niche for most phytoplankton and thus did not induce significant thermal stress and alterations in fatty acid profiles. Previous studies have shown that this temperature increase also did not notably change the phytoplankton species composition (Moss et al., 2003, Özen et al., 2013, Birk et al., 2020). Laboratory experiments have repeatedly demonstrated a warming effect on EPA and DHA proportions in algae, however, the temperature gradient has typically been considerably greater than that in the current study (Hixson and Arts, 2016, Nalley et al., 2018). Also, in the current study the data are presented as EPA and DHA concentrations and not as proportions, thus the total phytoplankton biomass strongly affects the values. This is apparent also from the model results; after the effect of diatoms were taken into account, phytoplankton biomass could explain an additional 14 % of the variation in EPA and DHA concentrations. Overall, our results indicate that in temperate shallow lakes increased loading of nutrients will most likely affect phytoplankton EPA and DHA

concentrations indirectly, i.e., by altering phytoplankton community structure and biomass.

Higher nutrient concentrations increased phytoplankton biomass in spring and summer but nutrient effects on phytoplankton EPA and DHA concentrations were highly variable. The within group variation of EPA and DHA in the N + P addition mesocosms in spring was attributed to variable responses of the phytoplankton community structure and biomass to the nutrient additions. Mesocosms which were dominated by diatoms in spring had high EPA concentrations while those dominated by cyanobacteria/chlorophytes had lower EPA concentrations. The differences in the abundance of diatoms and cyanobacteria/chlorophytes among mesocosms indicate system-specific or even stochastic differences in the responses to increased nutrient loading. The climate scenarios did not explain the high variation in phytoplankton community structure (inferred from fatty acid profiles) or the concentrations of EPA and DHA in phytoplankton. In these highly controlled mesocosms the variation may in part be attributed to the presence of macrophytes, namely *Elodea canadensis* and



**Fig. 2.** A dbRDA plot based on the DistLM model of the concentrations of EPA and DHA in mesocosms with different nutrient additions and climate scenarios sampled in May or July. Data were log-transformed prior to the analysis. Model results are presented in Table 4. Individual predictors with strong ( $r > 0.5$ ) relationship with the dbRDA axes are presented in the plot.

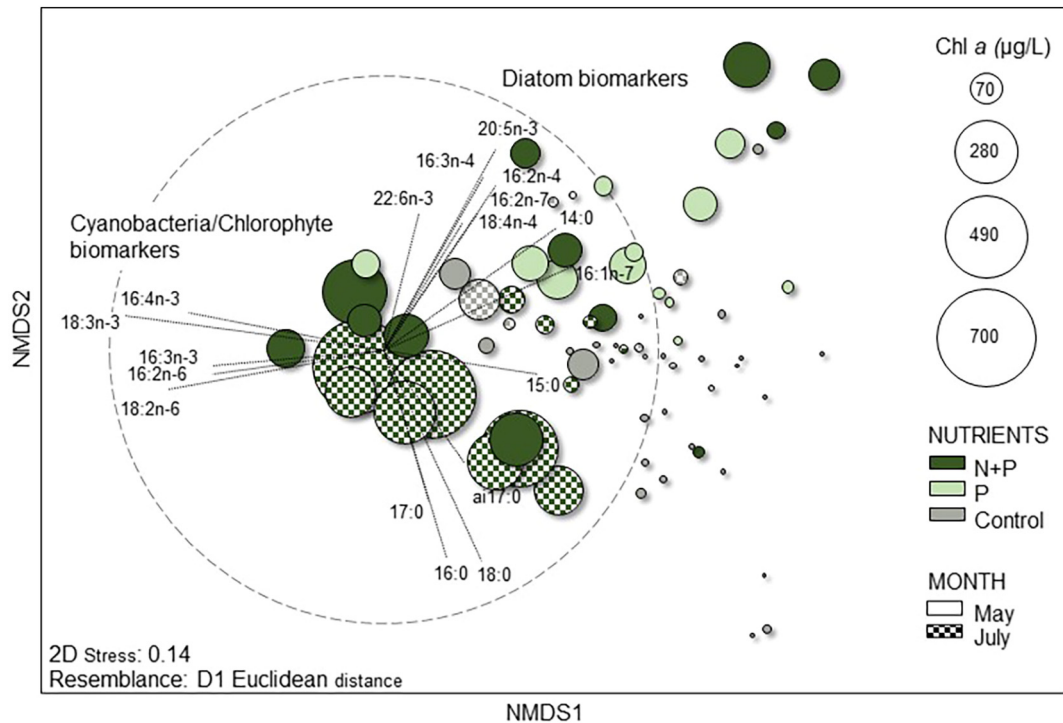


Fig. 3. NMDS based on sestion fatty acid profiles (w%). Vector overlays indicate the strength of the relationship between the variables and the NMDS axes.

*Potamogeton crispus* (Pacheco et al., 2021), and filamentous green algae, as well as grazers, such as snails and zooplankton, which were not controlled in the experiment. The proportion of macrophytes and filamentous green algae, which compete for nutrients with phytoplankton, varied between mesocosms, as after 15 years the mesocosms have developed differently as is also the case for natural lakes. Despite the high within group variation in spring, significant seasonal differences were observed, i.e., the concentrations of EPA were low in summer in all mesocosms with nutrient additions. In the summer, the high phytoplankton biomass corresponded with low EPA and DHA concentrations. This is likely due to cyanobacteria dominance (Filiz et al., 2020) and a smaller contribution of diatoms to the phytoplankton community, as indicated by the decreased proportion of diatom-specific biomarkers. Higher summer phytoplankton biomass (quantity) did not compensate for the poorer phytoplankton quality. The seasonal shift from diatoms to cyanobacteria/chlorophyte dominance was also indicated by the n-3/n-6 ratio, which declined from May to July in the nutrient addition mesocosms. Note, that climate scenario did not affect the phytoplankton n-3/n-6 ratio, in any of the nutrient treatments or seasons, even though

this ratio in laboratory experiments has been shown to strongly respond to temperature (de la Rosa et al., 2020; Renaud et al., 2002). This indicates that direct ecophysiological adaptations to higher temperatures were likely not the main reason for the decline of n-3/n-6 ratio in summer in these mesocosms with highly complex phytoplankton assemblages. Additionally, our results revealed that the spring diatom bloom may be highly important for the annual supply of EPA and DHA in lakes and a decline in spring diatom biomass or shift to a more chlorophyte/cyanobacteria dominated spring community may negatively affect secondary production and the levels of EPA and DHA in fish (Müller-Navarra et al., 2000; Taipale et al., 2016). Diatoms are rich in EPA, which may comprise 7–30 % of their total fatty acids (Dunstan et al., 1993; Taipale et al., 2013). Diatoms are generally fast growing and often dominate at non-limiting turbulent conditions (Egge, 1998), and thus the EPA pool may be high during spring diatom blooms. The size distribution of diatoms is important for determining the trophic transfer of EPA to consumers; large-sized phytoplankton and phytoplankton colonies are poorly grazed by smaller cladoceran zooplankton, and thus may create a bottleneck for the trophic transfer of EPA, as suggested in lakes which were dominated by the large-sized nuisance alga, *Gonyostomum semen* (Strandberg et al., 2020). Diatoms are often sensitive to settling in the water column because their frustules are 3 % denser than water and many diatom taxa form large, heavy colonies, and thus require moderate turbulent mixing to remain in the photic zone (Huisman et al., 2004).

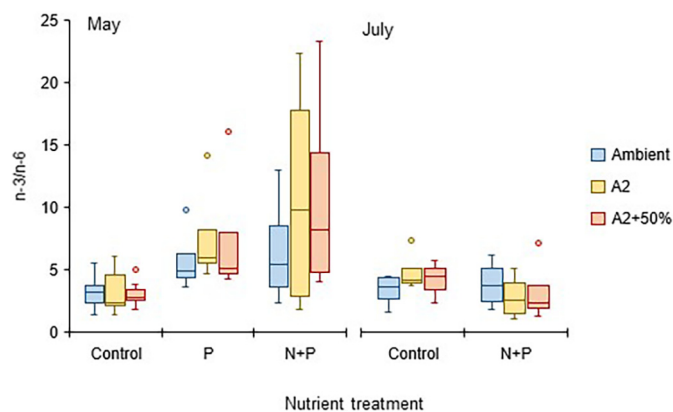


Fig. 4. Sestion n-3/n-6 ratio in May and July in mesocosm with different nutrient treatments: control, i.e., low nutrient condition, and P or N + P additions and climate scenarios.

Nutrient limitation constrained phytoplankton biomass and, thus, the EPA and DHA concentrations were always modest (mean values <9 µg/L) in the mesocosm without additional nutrient additions (only input from groundwater). Nutrient effects on algae metabolism and biochemistry have been widely studied and, in most algae, nutrient depletion, particularly N-limitation, has been connected to increased lipid accumulation, however, the increase occurs mainly for SFA and MUFA, and not for PUFA (Juneja et al., 2013, Fernandes et al., 2016, Yaakob et al., 2021). This is in accordance with the current study showing that the nutrient effect on the EPA and DHA concentrations could mainly be attributed to changes in phytoplankton biomass and community. Although, our fatty acid biomarker approach cannot differentiate cyanobacteria and chlorophytes, the low EPA and DHA concentrations in the low nutrient tanks are likely



due to chlorophyte dominance, and not cyanobacteria, as indicated by a previous study of these tanks (Filiz et al., 2020). Also, the n-3/n-6 ratio was stable in the nutrient depleted mesocosms (control), regardless of the sampling season or climate scenario. The climate scenarios did not affect the phytoplankton EPA and DHA concentrations in the low nutrient or the N + P addition mesocosms. This is in accordance with previous mesocosm and multiscale studies showing that temperature-induced effects on phytoplankton biomass or community structure were modest compared to the effects of nutrients (Moss et al., 2003, Özen et al., 2013, Birk et al., 2020). However, the climate scenarios had a significant effect on phytoplankton EPA and DHA concentrations in the high loading mesocosm in the period with only P additions, indicating a possible interaction between the N:P ratio and climate-induced warming on the phytoplankton production of EPA and DHA. Data from lakes in boreal and sub-arctic regions have also suggested that temperature is not a proximate driver for seston PUFA content, but rather that the lake PUFA dynamics were more directly controlled by the N:P ratio and browning (Lau et al., 2021), which, however, are affected by climate change.

The climate scenario, i.e., warming, was not an important driver for the phytoplankton fatty acid profiles or community composition, and consequently the concentrations of EPA and DHA. Warming and nutrients may affect phytoplankton community structure by shifting the community toward taxa with higher growth optimum and/or efficient nutrient uptake. The thermal tolerance traits vary among taxa, but generally cyanobacteria have high temperature optima for growth, providing a competitive advantage at higher temperatures (Carey et al., 2012; Nalley et al., 2018). Decreased vertical mixing and increased thermal stratification alter phytoplankton community structure and have been noted to be integral in the formation of cyanobacteria blooms (Boehrer and Schultze, 2008; Joehnk et al., 2008). It is likely that global warming will result in longer and more pronounced thermal stratification in many lakes (Rühdland et al., 2015); potentially decreasing the concentrations of EPA and DHA in phytoplankton more than suggested by the results obtained from fully mixed mesocosms. Also, the temperature effects on phytoplankton are complex and interlinked with other environmental factors, such as nutrient availability (O'Connor et al., 2009). In nutrient depleted conditions resource availability constrained primary production and total standing biomass in all climate scenarios (O'Connor et al., 2009). This is in accordance with the current study, in which the concentrations of EPA and DHA were low in nutrient depleted conditions regardless of the climate scenario. We do not have zooplankton data for the experiment to evaluate the importance of temperature-induced grazing pressure as a driver for the phytoplankton community structure and the concentrations of EPA and DHA. However, it is likely that the effects of nutrient loading on EPA and DHA concentrations in natural phytoplankton communities will be highly dynamic and affected by trophic interactions, such as competition and grazing pressure (İşkin et al., 2020; Zhang et al., 2021).

## 5. Conclusions

Our results indicate that environmental change, namely eutrophication, will mostly affect phytoplankton EPA and DHA concentrations indirectly by altering the phytoplankton community composition and biomass. This also implies that the direct ecophysiological adaptations of phytoplankton to temperature and/or nutrient availability are less important drivers for the overall EPA and DHA concentrations than changes in the phytoplankton community structure. This is most likely due to ecological factors, such as interspecific competition and grazing pressure, which seem to have a stronger selective force on phytoplankton communities, and thus EPA and DHA concentrations. Extensive physiological and metabolic acclimation processes to changing conditions may not be sustainable for longer time periods in competitive conditions. Large system-specific differences in the responses to increased nutrient loading were observed in phytoplankton EPA and DHA concentrations, which suggests that responses in natural lakes to increased nutrient loading are likely variable and depended on other environmental as well as ecological drivers. The results of this study

can be applied to improve our understanding of the proximate reasons for the changes in the EPA and DHA concentrations in shallow lakes, which may have practical applications for lake management purposes and evaluations of the availability of EPA and DHA to upper trophic level consumers, such as fish. For instance, our results indicate that the nutrient-induced increases in the dominance of cyanobacteria likely decrease the availability of EPA and DHA to upper trophic level consumers including fish, as also suggested by Taipale et al. (2016).

## CRediT authorship contribution statement

Conceptualization: US, MH, MTB; Investigation: US, MH, JS; Methodology: US, MH, EET, TAD, EJ; Writing – original draft: US; Writing – review & editing: US, MH, JS, EET, TAD, EJ, MTB.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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U.S., M.H., M.B. conceived the study; E.E.L., T.A.D. and E.J. were responsible for the design, setup and running of the mesocosms; U.S., M.H. and J.S. collected the samples; U.S. and M.H. analyzed the fatty acids; U.S. analyzed the data and wrote the first draft. All authors revised the manuscript and approved the final version. This study was financially supported by Academy of Finland (grants #338261 and #346541 to U.S., #315163 to M.H., #296918 and #307238 to J.S., and #310450 to P. Kankaala) and the AQUACOSM and AQUACOSM-plus project Phytolipids funded by the European Union's Horizon 2020 research and innovation programme under grant agreements Nos. 731065 and 871081. E.J. was also supported by the TÜBITAK program BİDEB2232 (project 118C250) and E.J. and T.A.D. by AnaEE Denmark. T.A.D. and E.E.L. were also supported by project: GREENLAKES (No. 9040-00195B) and PONDERFUL, POND Ecosystems for Resilient FUTURE Landscapes in a changing climate, (Grant agreement ID: 869296) H2020 LC-CLA-2018-2019-2020

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2022.157001>.

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