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Marco Calderini

Environmental Change Effect on Quality of Production in Boreal Lakes

UNIVERSITY OF JYVÄSKYLÄ
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ABSTRACT

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Phytoplankton occupy a key position in aquatic food webs and their response to environmental changes can significantly impact the availability of energy and nutrients for the higher trophic levels. In this thesis, laboratory experiments, together with environmental sampling, were used to study how browning, eutrophication, and warming of boreal lakes affect the quality of phytoplankton production and its ramifications for food webs. Quality was centred on polyunsaturated fatty acids (PUFA), with an emphasis on eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as these PUFAs are required for the growth and reproduction of consumers and are not synthesized by all phytoplankton species. Browning-driven decreases in light availability led the mixotrophic cryptophyte *Cryptomonas* sp. to increase reliance on bacterial-derived organic carbon over photosynthesis to sustain growth. However, this change in carbon utilization did not impact *Cryptomonas* sp. PUFAs, suggesting that browning will decrease primary production without affecting phytoplankton quality. Warming and eutrophication had opposing effects on the PUFA proportion of ten boreal phytoplankton species (from six different groups). Moreover, EPA and DHA production had a species-specific response to these environmental changes. Consequently, lakes with different nutrient levels may respond differently to warming with phytoplankton quality being mostly determined by phytoplankton community composition. In Finnish boreal lakes, eutrophication led to higher phytoplankton, zooplankton, and fish biomasses while altering the whole food web community. Volumetric primary production saturated at high phytoplankton biomass while EPA volumetric production responded logarithmically to eutrophication. Primary and EPA productivity (production per seston biomass) had unimodal responses to eutrophication. DHA volumetric production and productivity varied largely with eutrophication but were best described by unimodal models. Overall, eutrophication impaired EPA and DHA transfer from phytoplankton into zooplankton and fish.

Keywords: Browning; eutrophication; phytoplankton; production; quality; warming.

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TIIVISTELMÄ

Calderini, Marco
Ympäristömuutosten vaikutus boreaalisten järvien tuotannon laatuun
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Kasviplankton on avainasemassa järvien ravintoverkossa, ja sen reagointi ympäristön muutoksiin voi merkittävästi vaikuttaa korkeampien trofiatasojen energian ja ravintoaineiden saatavuuteen. Tässä väitöskirjatyössä laboratoriokokeiden ja ympäristönäytteiden avulla selvitettiin, miten boreaalisten järvien veden värin tummuminen, rehevöityminen ja lämpeneminen vaikuttavat kasviplanktontuotannon laatuun ja lopulta koko ravintoverkkoon. Laatu keskittyi monityydyttymättömiin rasvahappoihin (PUFA), painottaen eikosapentaeenihappoa (EPA) ja dokosaheksaeenihappoa (DHA), koska näitä PUFA-yhdisteitä tarvitaan kuluttajien kasvuun ja lisääntymiseen, eivätkä kuluttajat pysty niitä itse valmistamaan. Tummumisen aiheuttama valon saatavuuden heikkeneminen lisäsi miksotrofisen nielulevän (*Cryptomonas* sp.) riippuvuutta orgaanisesta hiilestä yhteyttämisen sijaan. Tämä muutos hiilen käytössä ei kuitenkaan vaikuttanut monityydyttymättömien rasvahappojen määrään nielulevässä. Lämpenemisellä ja rehevöitymisellä oli vastakkaisia vaikutuksia monityydyttymättömien rasvahappojen määrään kymmenessä boreaalisen järven kasviplanktonlajissa. Lisäksi EPA- ja DHA-tuotannolla oli lajikohtainen vaste tutkittuihin ympäristömuutoksiin. Tästä johtuen järvet, joiden ravinnetasot vaihtelevat, voivat reagoida lämpenemiseen eri tavoilla, ja kasviplanktonin laatu määräytyy pääasiassa kasviplanktonyhteisön mukaan. Suomen boreaalisissa järvissä rehevöityminen johti kasviplanktonin, eläinplanktonin ja kalojen biomassan kasvuun ja muutti koko ravintoverkkoyhteisöä. Volumetrisen perustuotannon (litraa kohti) kasvu pysähtyi kasviplanktonbiomassan kasvaessa volumetrisen EPA-tuotannon vasteen ollessa logaritminen. Biomassaan suhteutetuilla perus- ja EPA-tuotannolla oli yksihuippuiset suhteet rehevöitymisen kanssa. DHA:n volumetrinen ja biomassaan suhteutettu tuotanto vaihtelivat huomattavasti ravinnegradientissa, mutta suuntauksia kuvasivat parhaiten yksihuippuiset mallit. Kaikkiaan rehevöityminen heikensi EPA:n ja DHA:n siirtymistä kasviplanktonista eläinplanktoniin ja kaloihin.

Avainsanat: Kasviplankton; laatu; lämpeneminen; rehevöityminen; ruskettuminen; tummuminen; tuotanto.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I–III. The responsibilities and contributions of co-authors for each publication are explained in Table 1.

I Calderini M.L., Salmi P., Rigaud C., Peltomaa E. & Taipale S.J. 2022. Metabolic plasticity of mixotrophic algae is key for their persistence in browning environments. *Molecular Ecology* 31: 4726–4738.

II Calderini M.L., Pääkkönen S., Salmi P., Peltomaa E. & Taipale S.J. 2023. Temperature, phosphorus, and species composition will all influence phytoplankton production and content of polyunsaturated fatty acids. *Journal of Plankton Research* 45: 625–635.

III Calderini M.L., Kahilainen K.K., Estlander S., Peltomaa E., Piro A., Rigaud C., Ruuhijärvi J., Salmi P., Vesterinen J., Vuorio K. & Taipale S.J. Eutrophication effect on production and transfer of omega-3 fatty acids in boreal lake food webs. Submitted manuscript.

TABLE 1 Author contribution to original papers. Author abbreviations: AA = All authors, AP = Alex Piro, CR = Cyril Rigaud, EP = Elina Peltomaa, JR = Jukka Ruuhijärvi, JV = Jussi Vesterinen, KKK = Kimmo K. Kahilainen, KV = Kristiina Vuorio, MLC = Marco L. Calderini, PS = Pauliina Salmi, SP = Salli Pääkkönen, SJT = Sami J. Taipale, and SE = Satu Estlander.

	I	**II**	**III**
Planning	EP, MLC, PS, SJT	MLC, PS, SP, SJT	MLC, SJT
Laboratory work	CR, MLC, PS	MLC, PS, SP	MLC, KV, SE
Field work	-	-	AP, CR, JR, JV, MLC
Data analysis	MLC	MLC	MLC
Manuscript draft	MLC	MLC	MLC
Manuscript revisions	AA	AA	AA
Correspondence	MLC	MLC	MLC

1 INTRODUCTION

1.1 Phytoplankton

Phytoplankton, a diverse and polyphyletic group of photosynthetic organisms that live suspended in aquatic ecosystems (Falkowski 2004), span eight phyla (Ruggiero *et al.* 2015) and encompass eukaryotic and prokaryotic organisms that exhibit variations in morphology, organization (free-living or associated to other cells), metabolism, and biochemical composition (Lubchenco and Cubit 1980, Ahlgren *et al.* 1992, Reynolds 2006, Falkowski and Raven 2007, Peltomaa *et al.* 2017). Despite accounting for a small share of Earth's photosynthetic biomass, phytoplankton are responsible for over 45 % of the planet's annual net fixation of carbon dioxide (CO_2) into biomass (Field *et al.* 1998). In addition to their role in carbon cycling, phytoplankton are central to aquatic ecosystems as they provide most of the energy and nutrients upon which food webs rely (Reynolds 2006). Food webs are hierarchical arrangements that depict the relative positions of organisms in a food chain. They consist of trophic levels, which represent the number of steps required to locate an organism starting from the base of the chain. Classical pelagic food webs begin with phytoplankton, from where energy and nutrients are transported to herbivorous zooplankton and further to planktivorous fish (Sieburth *et al.* 1978). The energy transfer efficiency of the first trophic interaction (phytoplankton-zooplankton) depends on phytoplankton edibility and nutritional quality (Elser *et al.* 2000, Dickman *et al.* 2008). Thus, the relative prevalence of different species, collectively referred to as communities, and their growth, are key determinants of aquatic food web functioning.

Primarily, phytoplankton growth is considered resource-controlled (bottom-up) by light quality and quantity (Bergström *et al.* 2003, Karlsson *et al.* 2009, Edwards *et al.* 2015), temperature (Smol *et al.* 2005), and nutrients (Hasler 1947, Zohary 2004, Keva *et al.* 2020). Traits related to light energy harvesting such as chlorophyll content and type (a, b, c, and d), as well as accessory pigment

composition (carotenoids and phycobilins), separate ecological niches of different phytoplankton species across light availability gradients (Litchman and Klausmeier 2008). Despite variability among species, major phytoplankton groups tend to have characteristic ranges of these light harvesting traits (Richardson *et al.* 1983, Geider *et al.* 1998). For example, diatoms, dinoflagellates, and cyanobacteria can harvest light energy very efficiently, allowing them to dominate low-light environments over groups requiring high-light intensities such as green algae (Richardson *et al.* 1983, Geider *et al.* 1998, Litchman and Klausmeier 2008). The uptake and utilization of essential macro- and micronutrients such as phosphorus, nitrogen, iron, zinc, and others are characterized by cell surface-to-volume ratio and enzyme kinetics, which directly condition the environmental nutrient requirements of phytoplankton species (Eppley *et al.* 1969, Sommer 1981, Litchman and Klausmeier 2008). Smaller species tend to have higher growth rates and higher affinity for limiting nutrients than larger species, while the latter have larger nutrient storage capacity, and are more resistant to predation (Litchman *et al.* 2007). In addition, smaller phytoplankton tend to be more photosynthetically efficient and fixate more CO_2 per biomass than larger species, highlighting their relevance in the carbon cycling of water ecosystems (Li 1994, Irion *et al.* 2021). Altogether, smaller species can outcompete larger species in oligotrophic systems where rapid assimilation of nutrients confers a competitive advantage. However, migration to more resource-rich segments of the water column (propelled via flagella) or the ability to feed heterotrophically can help bypass the ecological restrictions imposed by size. When an organism can utilize both light energy (phototrophy) and organic carbon (heterotrophy) to sustain growth, it is referred to as mixotrophic. Most commonly, mixotrophic phytoplankton obtain organic carbon via osmosis from the surrounding environment (osmotrophy) or by engulfment of particulate matter, such as bacteria (phagotrophy) (Stoecker *et al.* 2017, Bock *et al.* 2021). The occurrence of mixotrophy is well documented in phytoplankton groups considered poor competitors for inorganic nutrients such as dinoflagellates and cryptophytes (Smayda 1997, Bergström *et al.* 2003, Litchman *et al.* 2007). Conversely, the capacity of mixotrophy has been observed in species of phylogenetically distant phytoplankton groups such as diatoms, raphidophytes, haptophytes, golden algae, cyanobacteria, and green algae (Lewitus *et al.* 1991, Bernát *et al*. 2009, Jeong 2011, Sanders and Gast 2012, Kamikawa *et al.* 2015, Gerea *et al.* 2016, Stoecker *et al.* 2017, Bock *et al.* 2021). All physiological traits described thus far that are involved in photosynthesis, growth, and resource acquisition are highly dependent on temperature (Eppley 1972, Raven and Geider 1988, Dell *et al.* 2011). This dependency translates into a range of suitable temperature for the growth of phytoplankton, which is defined as a species' thermal niche (Thomas *et al.* 2012). Despite within species variations, diatoms tend to have lower minimal growth temperatures than dinoflagellates and raphidophytes, while green algae and cyanobacteria have overall higher thermal niches (Seip and Reynolds 1995, Litchman and Klausmeier 2008, Chen 2015).

Theoretically, with low habitat variability, phytoplankton should reach an equilibrium where communities are composed of species with suitable traits to the abiotic and biotic environment (Margalef 1978). However, aquatic ecosystems are subject to seasonal and long-term variation in environmental conditions, making phytoplankton temporally dynamic (Margalef 1978, Zohary 2004, Smol *et al.* 2005, Reynolds 2006). In high latitudes regions of the globe such as the boreal zone, seasonal fluctuations of climatic conditions are pronounced, with strong annual oscillations in light availability and temperature. Within boreal aquatic ecosystems, lakes are particularly susceptible to environmental variability as their physical and chemical properties rapidly respond to environmental change (Adrian *et al.* 2009). Environmental-driven effects on lake phytoplankton abiotic ecological boundaries (Adrian *et al.* 2009) include modifications of nutrient inputs driven by changes in precipitation patterns and anthropogenic activities in catchment areas, as well as changes in the ice-covered duration and temporal distribution. This link between environmental conditions and lake phytoplankton dynamics is of particular interest to understand how climatic variation and anthropogenic activity affect phytoplankton fixation of CO_2, community composition, and their cascading effects on food webs.

1.2 Environmental change in boreal lakes

Anthropogenic activity, in the form of climate change and land use in catchment areas, is having a profound impact on surface waters throughout the boreal zone. Over the last decade, lakes in this region of the world have been rapidly warming (Schneider and Hook 2010, O'Reilly *et al.* 2015, IPCC 2021) while receiving increasing concentrations of nutrients and coloured dissolved organic carbon (DOC) (Björnerås *et al.* 2017, Creed *et al.* 2018, Räike *et al.* 2020, Blanchet *et al.* 2022). Concomitant to alterations in temperature, climate-driven increases in the frequency and intensity of precipitations (de Wit *et al.* 2016, Ruosteenoja *et al.* 2016, IPCC 2021) combined with shorter ice-covered periods (Magnuson 2000, Adrian *et al.* 2009), are expected to facilitate the inflow of nutrients (mostly phosphorus) and DOC from catchments promoting eutrophication and further browning of waters, respectively (Björnerås *et al.* 2017, Creed *et al.* 2018, Räike *et al.* 2020, Blanchet *et al.* 2022, Isles *et al.* 2023). This transport of nutrients and DOC is particularly influential in lakes with catchments dominated by intense agricultural and peatland-forestry activities (Räike *et al.* 2020, Finér *et al.* 2021). Warming, eutrophication, and browning of waters will likely alter nutrient cycling, phytoplankton community composition, and food web structure and functioning, leading lakes towards new ecological states (Smol *et al.* 2005, Smol and Douglas 2007, Jeppesen *et al.* 2010, Creed *et al.* 2018).

By the end of the 21st century, air temperatures in boreal Fennoscandia are estimated to increase between 2–7 °C (IPCC 2014: RCP2.6—RCP8.5), which will likely elevate surface water temperatures simultaneously. An increase in lake water temperature directly affects organism-level metabolic rates and leads

communities towards warm-adapted species (Smol and Douglas 2007, Hood *et al.* 2018, Keva *et al.* 2020). At the same time, elevated temperatures are associated with higher terrestrial organic carbon production, decomposition, and export to surface waters (Finstad *et al.* 2016). Analysis of marine and freshwater phytoplankton communities across temperature gradients suggests an increased abundance of cyanobacteria over groups commonly observed in colder waters such as diatoms (Jöhnk *et al.* 2008, Morán *et al.* 2010, Keva *et al.* 2020). Eutrophication driven by increases in phosphorus supply, one of the most limiting nutrients for phytoplankton (Bergström and Jansson 2006, Schindler 2012), boosts their growth and total food web biomass (Hasler 1947, Schindler 1977, Qin and Shen 2019, Keva *et al.* 2020) while altering community composition of all trophic levels (Vollenweider *et al.* 1974, Jeppesen *et al.* 2000, Hayden *et al.* 2017, Keva *et al.* 2020). Studies have shown that as the concentration of total phosphorus (TP) increases in aquatic environments, the relative abundance of cryptophytes and golden algae decreases, while euglenoids, green algae and cyanobacteria increase (Watson *et al.* 1997, Taipale *et al.* 2016, Keva *et al.* 2020). Elevated TP concentrations are also linked to an increase in the frequency, duration, and intensity of cyanobacteria blooms with toxic or deleterious properties (Heisler *et al.* 2008, Glibert and Burford 2017, Vuorio *et al.* 2020a), particularly when in combination with high temperatures (Dai *et al.* 2023). Browning of waters increases vertical attenuation of photosynthetically active radiation, narrowing the photic zone (Creed *et al.* 2018, Kritzberg *et al.* 2020, Blanchet *et al.* 2022). Once light becomes limiting (> 5 mg DOC l^{-1}, Seekell *et al.* 2015), browning reduces the photosynthetic capacity of lakes, shifting the balance from phytoplankton to bacterial production, and reducing energy transfer efficiency through aquatic food webs (Hessen 1998, Creed *et al.* 2018, Blanchet *et al.* 2022). Phytoplankton communities in high DOC lakes present less overall diversity (Jones 1992) and tend to be dominated by mixotrophic cryptophytes and raphidophytes (specifically the species *Gonyostomum semen*) (Bergström *et al.* 2003, Deininger *et al.* 2017, Hagman *et al.* 2020). This increase in the share of mixotrophic phytoplankton suggests an alteration of the carbon and oxygen cycling of lakes driven by higher phytoplankton heterotrophy.

1.3 Phytoplankton nutritional quality

Edibility and nutritional quality of prey items constrain the efficiency of nutrients and energy transfer during trophic interactions (Elser et *al.* 2000, Dickman *et al.* 2008). When edibility requirements are satisfied, the transfer of energy and nutrients from phytoplankton to zooplankton has typically been characterized by phytoplankton's carbon and macronutrient (e.g., phosphorus and nitrogen) stoichiometry (Elser et *al.* 2000). Nevertheless, micronutrients are increasingly recognized as important predictors of energy transfer at the phytoplankton–zooplankton interface (Müller-Navarra *et al.* 2000, Peltomaa *et al.* 2017). Among phytoplankton micronutrients, essential amino acids, sterols, and specifically

fatty acids (FAs) have been described as required for efficient energy transfer to zooplankton (Müller-Navarra *et al.* 2000, Martin-Creuzburg *et al.* 2009, Peltomaa *et al.* 2017). FAs are a subclass of lipid biomolecules required for proper cellular function. Free FAs are aliphatic chains with a carboxylic group at one end, and their classification is based on the presence and position of carbon-to-carbon unsaturation. Saturated FAs lack unsaturation, monounsaturated FAs have one unsaturation, and polyunsaturated FAs (PUFAs) have two or more unsaturations. The distance in units of carbon of the first double bond in reference to the methyl end (also called omega carbon) is used to further classify FAs into different omega categories. FAs are involved in diverse cellular functions such as structural (e.g., phospholipids), energy metabolism (e.g., direct oxidation, storage as triglycerides), and signalling (e.g., eicosanoids) (Samuelsson 1983, Khozin-Goldberg 2016). As constituents of membrane lipids, the structural diversity of FAs provides numerous arrangements for fine-tuning membrane fluidity and permeability, thus controlling membrane function (Klose *et al.* 2012). Environmental changes that increase membrane fluidity (e.g., higher temperatures) commonly lead to a decrease in the unsaturation degree of membrane FAs to maintain homeostasis (Sinensky 1974). Moreover, since phosphorus is also necessary for the synthesis of phospholipids, changes in the supply of this nutrient can lead an organism to modify the type of structural lipids being used to assemble membranes (Van Mooy *et al.* 2009), indirectly affecting which FAs are integrated into these structures.

FAs are not equally distributed among phytoplankton (Ahlgren *et al.* 1992, Lang *et al.* 2011, Taipale *et al.* 2013), with long-chain (> 20 carbon atoms) omega-3 PUFAs such as eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3) being efficiently synthesized in cryptophytes, dinoflagellates, diatoms, and golden algae while green algae and cyanobacteria only contain trace amounts of these FAs (Müller-Navarra *et al.* 2004, Taipale *et al.* 2016). However, large variations in FA contents can be observed among species of the same phytoplankton group (Lang *et al.* 2011). EPA and DHA are essential for the development, neural function, and reproduction of consumers (Arts *et al.* 2001, Brett *et al.* 2009), and they are highly retained in aquatic food webs. Animals lack the necessary enzymes for de-novo synthesis of EPA and DHA (Henderson 1996, Cook and McMaster 2004), and therefore they rely on direct dietary acquisition or bioconversion from shorter PUFA precursors such as linolenic acid (LIN, 18:2ω-6) and α-linolenic acid (ALA, 18:3ω-3) to sustain physiological requirements (Twinning *et al.* 2016). Although bioconversion of LIN and ALA to long-chain PUFAs has been observed in invertebrates and fish (Kabeya *et al.* 2018, Ishikawa *et al.* 2019, Boyen *et al.* 2023), this process is considered inefficient and energetically expensive and is likely triggered only when dietary requirements cannot be met (Twinning *et al.* 2016). Given that FAs are not degraded during digestion (Dalsgaard *et al.* 2003, Iverson 2009), EPA and DHA are thought to be transferred directly from phytoplankton to higher trophic levels (Henderson 1996, Cook and McMaster 2004). Nevertheless, low availability of EPA and DHA in phytoplankton can be mitigated at higher trophic levels in eutrophic systems

(Kainz *et al.* 2017, Keva *et al.* 2020, Lau *et al.* 2021, Taipale *et al.* 2022), suggesting a lack of understanding of the flow of PUFAs across food webs.

Environmental factors that affect the composition of phytoplankton communities and the cellular contents of EPA and DHA are thought to control the availability of high nutritional value long-chain PUFAs in aquatic ecosystems (Kainz *et al.* 2004, Taipale *et al.* 2016). Currently, PUFA availability (particularly EPA and DHA) is expected to decrease in aquatic ecosystems due to climate-driven warming of waters (Hixson and Arts 2016, Colombo *et al.* 2019). Conservative estimates from modelling suggest reductions of phytoplankton synthesis of EPA and DHA of 8.2 % and 10–28 % (respectively) by the end of the 21st century (Hixson and Arts 2016, Colombo *et al.* 2019). Such reductions in the availability of these long-chain PUFAs could have detrimental effects on the fitness of zooplankton and fish (Kainz *et al.* 2004, Taipale *et al.* 2016). At the cellular level, EPA and DHA are considered highly fluidizing factors when integrated into membrane phospholipids (Valentine and Valentine 2004). Therefore, the reduction in their incorporation into membranes (and overall synthesis) would play a compensatory role to conserve membrane fluidity with higher temperatures (Sinensky 1974). Nevertheless, alterations in nutrient and light availability as a product of processes such as eutrophication and browning of waters could also affect the availability of these long-chain PUFAs in lakes by modulating phytoplankton communities (Bergström *et al.* 2003, Heisler *et al.* 2008, Marinov *et al.* 2010, Deininger *et al.* 2017, Vuorio *et al.* 2020a) and cellular contents of PUFAs (Van Mooy *et al.* 2009, Matsui *et al.* 2020). Reduction of available photosynthetically active light can alter EPA and DHA synthesis since these PUFAs participate in the acclimatation of photosynthetic membranes to varying light conditions (Valentine and Valentine 2004, Wacker *et al.* 2016). In addition, browning has been shown to increase the contribution of high EPA and DHA producing phytoplankton groups such as cryptophytes (Bergström *et al.* 2003, Deininger *et al.* 2017). Eutrophication enhances phytoplankton growth increasing volumetric concentrations of EPA and DHA (Taipale *et al.* 2016, Strandberg *et al.* 2022). Moreover, higher phosphorus availability in phosphorus limited lakes could indirectly affect the FA profiles of phytoplankton by altering the type and composition of membrane lipids (Van Mooy *et al.* 2009). Therefore, understanding how phytoplankton respond to the multivariate changes in environmental conditions provide insight into the future of PUFAs in lakes (Marinov *et al.* 2010).

1.4 Measurements of phytoplankton quality and production

Phytoplankton primary production is the rate at which energy is converted into biomass through the photosynthetic reduction of CO_2 into organic compounds (Falkowski and Raven 2007). The quality of production is therefore given by the nutritional properties of the produced organic compounds for consumers.

Herein, quality is discussed in the scope of FA nutritional quality of phytoplankton, specifically referring to EPA and DHA.

A wide variety of methodologies and units of measurement are used to study both phytoplankton quality and production, each presenting advantages and disadvantages when trying to make connections between phytoplankton and higher trophic levels. Gas-chromatography coupled with mass spectrometry is commonly used to study the absolute quantity and relative abundance of different FAs in aquatic samples. Seston FAs are used as a proxy to estimate phytoplankton quality for zooplankton (Müller-Navarra *et al.* 2000). However, interpreting seston quality in terms of volumetric (per l of water) or content (per mg of dry weight) availability can lead to different ecological interpretations. For example, seston EPA and DHA volumetric concentrations tend to be highest in eutrophic lakes (Strandberg *et al.* 2022), while seston EPA and DHA contents present high variability and are lowest in hyper-eutrophic lakes (Müller-Navarra *et al.* 2004, Taipale *et al.* 2019). This discrepancy in the interpretation of different units, combined with the lack of information about phytoplankton communities and their temporal dynamics, could potentially explain the mismatch observed in EPA and DHA contents between phytoplankton and higher trophic levels in eutrophic lakes (Kainz *et al.* 2017, Keva *et al.* 2020, Taipale *et al.* 2022).

Identifying phytoplankton communities via microscope or metatranscriptomics (Vuorio *et al.* 2020b) provides another approach to studying quality given the differential distribution of FAs across phytoplankton groups (Ahlgren *et al.* 1992, Lang *et al.* 2011). The contribution of different phytoplankton groups can then be used to make estimations about the quality for higher trophic levels (Przytulska *et al.* 2017, Weigel *et al.* 2023). Another approach combines community data and estimations of the FA content of different phytoplankton groups to quantitative estimate quality (Taipale *et al.* 2016). Community-based approaches are useful when trying to elucidate how environmental change selects for certain traits that alter phytoplankton communities. However, these approaches are constrained by the assumption that species belonging to the same phytoplankton group possess identical FA characteristics (content and relative abundance of FAs). This assumption is not valid, as highlighted by the observed variability in FA contents across species of the same phytoplankton group (Lang *et al.* 2011). Furthermore, the assumption that a phytoplankton species under different environmental conditions has the same FA characteristics neglects the potential for FA responses to environmental change. Overall, none of the methods described so far account for variation over time, which could provide information about how the supply of nutrients affects their transfer to higher trophic levels. FA content analysis and community composition can potentially be used to calculate quality of production when used in combination with and estimation of phytoplankton growth rate. An example of this is the estimation of daily production of EPA and DHA (commonly referred as daily gain) using FA content per cell and growth rate in phytoplankton monocultures (Taipale *et al.* 2020).

Incorporation of carbon heavier isotopes (^{13}C and ^{14}C) into phytoplankton biomass and oxygen exchange techniques are used to directly quantify lake production (Schindler *et al.* 1972, Underwood and Kromkamp 1999). Despite these approaches supply reliable data about the movement of inorganic carbon into phytoplankton, no information is obtained about where carbon is been allocated, limiting the applicability of these techniques in the study of quality of production under natural conditions. Compound-specific isotope analysis (CSIA) represents an alternative to bulk production measurements where biomolecules of interest are chromatographically separated and their isotopic ratio value (e.g., of carbon, nitrogen, or hydrogen) is individually measured (Dijkman *et al.* 2009, Lammers *et al.* 2016). Seston labelling experiments using ^{13}C-inorganic carbon and FA CSIA has been used to determine the growth rate of several phytoplankton groups in lakes (Dijkman *et al.* 2009). In addition, CSIA of FAs has helped characterize how phytoplankton group-specific production varies with lake depth (Lammers *et al.* 2016). The specificity of CSIA to measure FA-specific production represents a promising tool to study how quality of production explain the movement of EPA and DHA in aquatic food webs.

2 AIMS OF THE THESIS

The main aim of this thesis was to evaluate how climate-driven warming, eutrophication, and browning of boreal lakes affect phytoplankton production of high nutritional quality FAs and their transfer across food webs (Fig. 1).

In the first experiment, the aim was to identify the mechanisms behind the dominance of cryptophytes in high DOC lakes and the extended effects in production and availability of high nutritional quality FAs (I).

In the second experiment, the aim was to study the interacting effects of warming and eutrophication on the growth and FA composition of 10 phytoplankton species common to boreal lakes (II). The objective was to determine if the decrease of PUFAs caused by warming could be mitigated by phosphorus.

At last, environmental sampling was performed to assess how eutrophication impacts food web biomass, community composition, phytoplankton production and the quality of production, and how these changes are related to the movement of EPA and DHA in higher trophic levels (III).

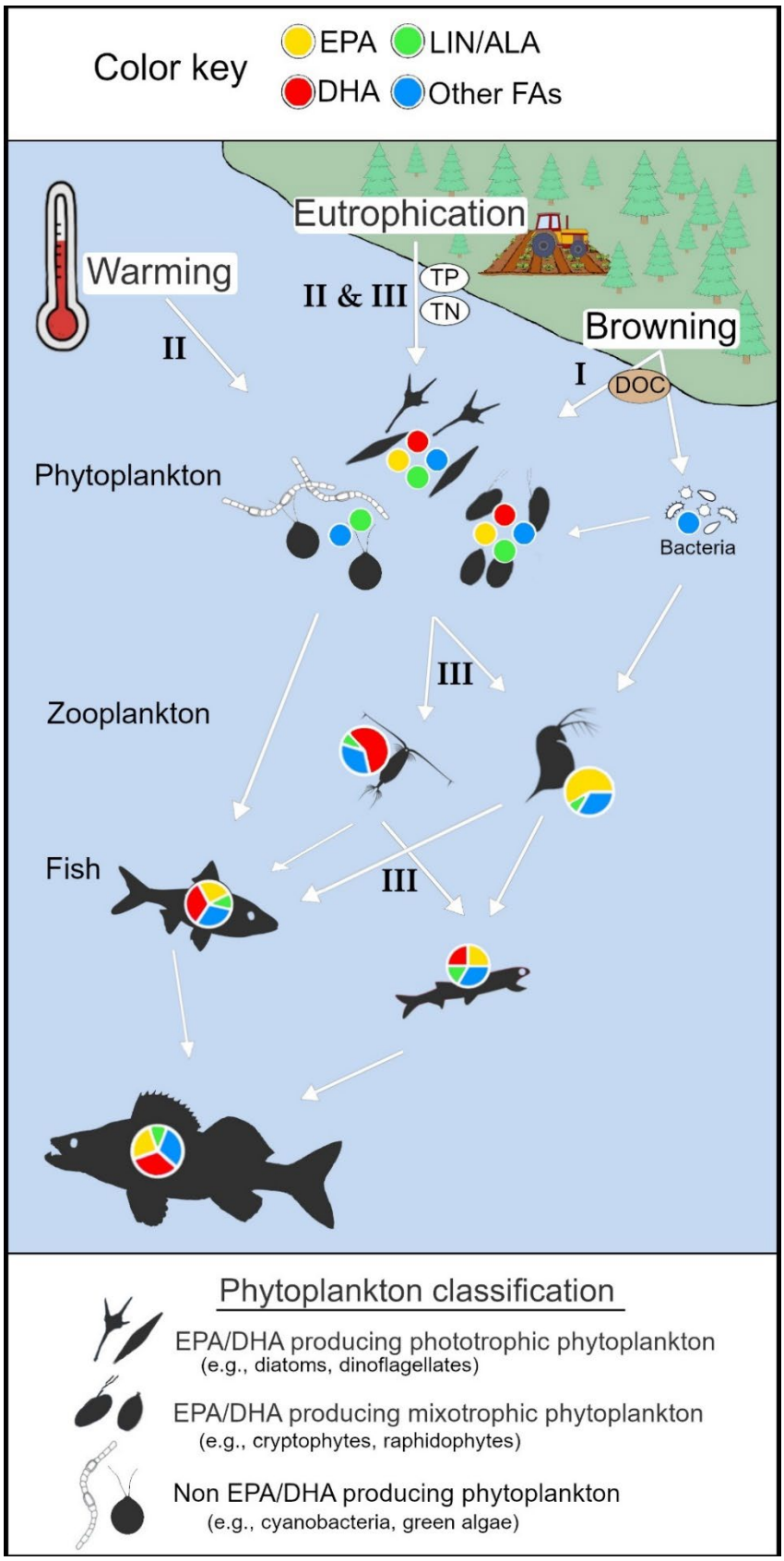

FIGURE 1 Graphical illustration of the main objectives of this PhD thesis. Pie diagrams were modified from Galloway and Budge (2020).

3 MATERIALS AND METHODS

3.1 Phytoplankton experiments

To test the responses of cryptophytes to high DOC (I), *Cryptomonas* sp. strain CPCC 336 (obtained from the Canadian Phycological Culture Centre), originally isolated from a clear-water lake, was cultured under phototrophic (Control), mixotrophic via osmotrophy (GLU), and heterotrophic (Dark) conditions. In addition to these treatments, five DOC conditions (concentrations: 1.5, 10, 30, 50 and 90 mg C l^{-1}) were tested. For the preparation of DOC treatments, a DOC mix (500 mg C l^{-1}) composed of lake water and a DOC extract obtained from peat was prepared and then added to phytoplankton cultures containing algal media. Temperature was maintained at 18 °C with a light–dark cycle of 12:12 h (light intensity 50–70 µmol quanta m^{-2} s^{-1}). To avoid carbon limitation during the extent of the experiment, 0.720 mg of carbon was added weekly to each culture. For phototrophic and DOC cultures, $NaHCO_3$ was added as a source of carbon while mixotrophic and heterotrophic cultures were supplied with glucose. Inorganic carbon added to all DOC cultures was enriched with 5 % ^{13}C-$NaHCO_3$ (Sigma-Aldrich, St. Louis, United States) for isotope analysis. The purpose of this was to study the incorporation of ^{13}C (fixation of inorganic carbon) into phospholipids.

To study the interacting effects of warming and eutrophication on the growth and FAs of phytoplankton (II), ten phytoplankton species common to boreal lakes from the groups golden algae (*Synura* sp. and *Uroglena* sp.), diatoms (*Cyclotella* sp. and *Melosira* sp.), cryptophytes (*Rhodomonas* sp.), dinoflagellates (*Peridinium cinctum*), green algae (*Chlamydomonas reinhardtii* and *Desmodesmus maximus*), and cyanobacteria (*Microcystis* sp. and *Synechococcus* sp.) were grown phototrophically in temperature-controlled growth chambers FH-130 (Taiwan Hipoint Taichung, Taiwan) using two phosphorus concentrations (LP: 0.65 µM and HP: 2.58 µM) and two temperatures (18 and 23 °C). Light–dark cycle was

12:12 h (light intensity 100–125 µmol quanta m^{-2} s^{-1}). Phosphorus was added weekly to avoid the effects of phosphorus depletion.

The phytoplankton growth media used was Modified Wright's Cryptophyte media (Guillard and Lorenzen 1972) with the pertinent mentioned alterations (I and II). Cell concentrations were followed every 2–3 day in all experiments using a flow cytometer Guava easyCyte HT (Luminex, Austin, United States).

To study how eutrophication affects phytoplankton total and FA specific production (III), two water samples (160–1000 ml) corresponding to 2 × Secchi depth water were incubated for 2 hours at 21 °C under constant light (70−96 µmol quanta m^{-2} s^{-1}) for each of the 12 studied lakes. Before incubation, 99 % ^{13}C-labeled $NaHCO_3$ (Sigma-Aldrich, St. Louis, United States) was added to a final concentration corresponding to 4 % of the total dissolved inorganic carbon in each lake (Dijkman *et al.* 2009). Differences in volume between lakes were a product of variations in particulate matter present in the water. After incubation, seston was filtered through 3.0 µm cellulose nitrate membranes and stored at –80 °C. The obtention of water samples and lake characteristics is described in detail in Section 2.5 "Field work".

3.2 Total lipid extraction and fatty acid analysis

Total lipids were extracted from freeze-dried samples using a solution of chloroform:methanol:water (4:2:1) as described by Folch *et al.* (1957). Sonication was then applied (10 min) to ensure disruption of samples and maximize lipid extraction. Phase separation of solvents was facilitated by centrifugation and lipid rich fraction was separated and evaporated under a nitrogen stream at 50 °C (I, III, and III). For the experiment studying the effect of DOC concentration on *Cryptomonas* sp. (I), two total lipid samples were obtained from each replicate of each treatment. One of the obtained total lipid samples was fractionated to obtain phospholipid-enriched lipid fractions (as described in Taipale *et al.* 2021) while the other sample was kept as total lipids. Fractionation was performed using solid-phase extraction with 500 mg silica cartridges Bond Elut LRC—SI (Agilent, Santa Clara, United States). Cartridges were preconditioned with chloroform before addition of the total lipid extract. Once lipids were absorbed by the cartridge, chloroform (8 ml) and then acetone (8 ml) were used to elute nonpolar lipids, and the phospholipid fraction was eluted with 8 ml methanol.

All non-fractionated total lipid samples (I, III, and III) were transesterified overnight with methanolic H_2SO_4 (1 %, v/v) at 50 °C. FA methyl esters were analysed with a gas chromatography-mass spectrometry Shimadzu Ultra (Shimadzu, Kyoto, Japan). FAs were identified by their specific ions together with retention times. FA peak areas were integrated, and concentration was estimated using calibration curves prepared with the FA standard mix 556C (Nu-Chek-Prep, Elysian, United States). FA concentrations were then corrected using the internal standard phospholipid FA C19:0 or free FA C23:0 (Larodan, Solna,

Sweden) (I, III, and III). Given that the DOC extract used during *Cryptomonas* sp. cultivation contained FAs (I), for FA percentage and content calculations only the algal ω-3 (18:3ω-3, 18:4ω-3, 20:4ω-3, 20:5ω-3 and 22:6ω-3) and ω-6 (18:2ω-6, 22:5ω-6) FAs were used.

3.3 Bulk isotope analysis

Phospholipid-enriched lipid fractions (I) obtained as described in Section 2.2 "Total lipid extraction and fatty acid analysis" were dried under a nitrogen stream at 50 °C. Dried lipids were then dissolved in methanol and gently transferred to a smooth-wall D4057 tin cup (Elemental Microanalysis, Okehampton, United Kingdom). Tin cups were left at room temperature until all solvent was evaporated. Lipid samples' $\delta^{13}C$ value was obtained using a Thermo Finnigan DELTAplusAdvantage mass spectrometer (Thermo Electron, Waltham, United States) connected to a FlashEA 1112 Elementar Analyser (Thermo Scientific, Waltham, United States). In addition to experimental treatments (DOC conditions), the DOC mix used to prepare the DOC treatments was filtered, freeze-dried and its $\delta^{13}C$ value was obtained. As internal standards during the analysis, birch leaves (*Betula pendula*) were used.

3.4 RNA preparation and transcriptomic analysis

Phytoplankton cells from treatments phototrophic (Control), mixotrophic (GLU), DOC1.5, DOC30 and DOC90 (mg C l^{-1}) (I) were collected by centrifugation after 14 days of cultivation. Cells were homogenized in BashingBead lysis tubes (0.1 and 0.5 mm) containing 700 µl of DNA/RNA Shield. Total RNA was extracted using a Chemagic 360 and the Chemagic Viral DNA/RNA 300 Kit H96 (Chemagen Technology, Baesweiler, Germany), purified using Zymo spin-columns (Zymo Research, Irvine, United States) and quantified using a NanoDrop (Thermo Fisher Scientific, Waltham, United States). RNA library generation, transcriptome sequencing, and posterior transcript annotation together with differential expression analysis were conducted by Novogene (Beijing, China). Shortly, transcriptome sequencing was carried out with a NovaSeq 6000 (Illumina, San Diego, United States). De-novo transcriptome assembly was done using trinity 2.6.6 (Grabherr *et al.* 2011) in the absence of a reference genome. Clustering of contigs was performed with corset 4.6 (Davidson and Oshlack 2014) to remove transcript redundance. Differential expression analysis was done using deseq2 (Love *et al.* 2014) to estimate p-values associated to expression changes between our phototrophic control and the rest of the treatments. Transcripts were considered differentially expressed when the change in expression between the treatment and the phototrophic control was at least 2-fold while having a significant p-value ($|Log_2 FoldChange| > 1$ and $p < .05$).

Functional annotation of differentially expressed transcripts was done matching transcript sequences to the Kyoto Encyclopedia of Genes and Genome (KEGG) Orthology (KO) database using KAAS (KEGG Automatic Annotation Server). Due to the lack of a reference genome, many differentially expressed transcripts matched to the same KO gene IDs. Therefore, we calculated the weighted average Log_2 fold-changes for each KO gene ID using the transcript abundance values.

3.5 Field work

Boreal lakes (12) from central and southern Finland across a phosphorus and nitrogen gradient were sampled during the summer season (July) of 2021 (I). Lake chemistry such as chlorophyll-a, TP, total nitrogen, and DOC were analysed from each sampled lake (Table 2). In addition, catchment characteristics were retrieved from the Finnish Environment Institute VALUE-tool which uses the CORINE land cover map (Table 2). Given that TP and total nitrogen were highly positively correlated (Pearson's correlation = 0.73, t = 3.39, p = 0.007), TP was utilized for plotting and modelling purposes as a metric of lake total nutrients.

TABLE 2 Physical characteristics, water chemistry, and catchment properties of studied lakes (III). Sampled lake names are given in the first column. Lake land area (LA), lake mean depth (MD), chlorophyll-a concentration (Chl-a), total phosphorus (TO), total nitrogen (TN), dissolved organic carbon (DOC), agricultural coverage of catchment (Agr.), and forested coverage of catchment (For.) are listed in table columns. Sampling of lakes was conducted in July 2020.

Lake	LA (km^2)	MD (m)	Chl-a ($\mu g\ l^{-1}$)	TP ($\mu g\ l^{-1}$)	TN ($\mu g\ l^{-1}$)	DOC ($\mu g\ C\ l^{-1}$)	Agr. (%)	For. (%)
Kukkia	47	5.23	5.1	11	509	6.9	3.8	63.8
Isojärvi	18.3	16.4	6.6	5	415	10.8	0	78
Korpijärvi	31.2	10	2.3	4	413	6	2.4	62.8
Tuusulanjärvi	6	3.2	35	76	965	7.8	26	33.2
Hulausjärvi	2.2	1.1	81	99	1318	12	10	58.9
Lohjanjärvi	122	12.7	22	37	791	9.3	15.8	54.1
Vesijärvi	108	6	8.2	26	542	5.9	17.8	43.2
Valkea-Kotinen	0.04	3	8.2	13	584	12.7	0	79.6
Majajärvi	0.03	4.6	26	58	1024	19	0	89.9
Pääjärvi	13.4	15	11	13	1573	12.3	14.9	61.2
Enäjärvi	5	3.5	131	140	1814	6.4	18.5	36.4
Vikträsk	1.87	4.49	40	50	644	10.8	22	48.9

For each studied lake, water samples were collected from the photic zone (0.3–9.0 m, 2 × Secchi depth of each lake) with a 5 l water sampler (Limnos Ltd, Ljubljana, Slovenia) for subsequent incubation (described in Section 2.1 "Phytoplankton cultivation experiments") and production measurement (described in Section 2.6 "Compound-specific isotope analysis"). Seston FA samples from the photic zone water were obtained by filtration through 3.0 µm cellulose nitrate membranes. In addition, phytoplankton samples were taken for

quantitative biomass analysis. Briefly, water from the photic zone was stored in Lugol solution (1 ml per 200 ml) and then analysed under an inverted microscope by applying the Utermöhl technique (Utermöhl 1958). When possible, the identification of phytoplankton was done to the species level. Biovolumes of identified phytoplankton were then converted to fresh weight biomass using taxa morphology-specific geometric formulas (Hillebrand *et al.* 1999). Biomass estimates were next used to obtain carbon contents by applying experimentally derived carbon-mass ratios (Menden-Deuer and Lessard 2000). Utilizing phytoplankton morphology metrics (widest dimension) and observed association between cells (colony and filamentous phytoplankton), phytoplankton were classified as edible (< 35 µm) and non-edible (≥ 35 µm) for zooplankton (Watson and McCauley 1988, Heathcote *et al.* 2016).

Zooplankton samples for FA analysis were obtained with plankton nets (100, 250 and 500 µm mesh sizes). Collected zooplankton was then manually sorted and identified to the species level when possible. Categorization of zooplankton was based on taxonomy and feeding strategy, the selected subgroups correspond to herbivorous Cladocera (*Alona*, *Bosmina*, *Ceriodaphnia*, *Chydorus*, *Daphnia*, *Diaphanosoma*, *Holopedium*, *Limnosida*), predatory Cladocera (*Leptodora*), Cyclopoida (*Cyclopoida nauplius*, *Cyclopoida kopepodiitti*, *Diacyclops*, *Megacyclops*, *Mesocyclops*, *Thermocyclops*), Calanoida (*Calanoida nauplius*, *Calanoida kopepodiitti*, *Eudiaptomus graciloides*, *Heterocope Limnocalanus*), Rotifera (*Anuraeopsis*, *Ascomorpha*, *Asplanchna*, *Brachionus*, *Chromogaster*, *Collotheca*, *Colyrella*, *Conochilus*, *Conochiloides*, *Filinia longiseta*, *Gastropus*, *Kellicottia*, *Keratella*, *Notholca*, *Ploesoma*, *Bipalpus*, *Polyarthra*, *Pompholyx*, *Synchaeta*, *Trichocerca*), and Chaoborus. In addition to samples for FA analysis, quantitative estimations of zooplankton biomass were performed. For this, whole water column water was collected with a 7.1 l water sampler (Limnos Ltd, Ljubljana, Slovenia) and pooled into one bucket (50 l). Pooled water was then sieved through a 50 µm mesh net and preserved in 70 % ethanol. Enumeration and measurement of length and width of zooplankton individuals (identified to species level) was carried out under an inverted microscope. Then, the carbon biomass of each species was estimated based on the weight of 30 individuals using species-specific carbon regressions (Bottrell *et al.* 1976, Vasama and Kankaala 1990). Zooplankton community composition analysis was carried out using the obtained taxon-specific carbon biomass (mg C l^{-1}).

Fish samples from each lake were collected following the European Standard (EN 14757:2005, Water quality – sampling of fish with multi-mesh gillnets) utilizing Nordic multi-mesh gillnets. Fish were collected from littoral, pelagic, and profundal habitats, and euthanized by cerebral concussion after been removed from the gillnets. Muscle tissue from the species perch (*Perca fluviatilis)*, ruffe (*Gymnocephalus cernua)*, roach (*Rutilus rutilus*) and smelt (*Osmerus eperlanus*) were collected for FA analysis.

Data related to fish biomass per unit of effort (BPUE) and community composition was retrieved from the Natural Resources Institute Finland (LUKE) database. This data corresponds to the summer sampling of the year 2021 for 11

of the studied lakes, while Lake Enäjärvi represents the 2019 sampling (latest available data). For fish community composition analysis, fish species were grouped as percids (perch, pikeperch, *Sander lucioperca* and ruffe), cyprinids (minnow, *Phoxinus phoxinus*, roach, white bream, *Blicca bjoerkna*, common bream *Abramis brama*, common bleak, *Alburnus alburnus*, blue bream, *Ballerus ballerus*, crucian carp, *Carassius carassius,* ide, *Leuciscus idus*, rudd, *Scardinius erythrophthalmus*, and tench, *Tinca tinca*), salmonids (landlocked salmon, *Salmo salar*, vendace, *Coregonus albula*, European whitefish, *C. lavaretus*) and others (smelt, pike, *Esox lucius*, burbot, *Lota lota*, common bullhead, *Cottus gobio*).

3.6 Compound-specific isotope analysis

Total lipids were extracted from incubated enriched (^{13}C) seston samples (Section 2.1 "Phytoplankton cultivation experiments", III) and natural seston samples (Section 2.5 "Field work", III), were transesterified as described in Section 2.2 "Total lipid extraction and fatty acid analysis". The FA-specific $\delta^{13}C$ values were obtained using gas chromatography-mass spectrometry (7890B GC, 5977B MS, Agilent, Santa Clara, United States) connected to an isotope ratio mass spectrometer Isoprime precisION (Elementar, Langenselbold, Germany). The $\delta^{13}C$ values FAs were manually calculated using background values and corrected for the esterified methyl group as described by Dijkman *et al.* (2009). For drift and linear correction of $\delta^{13}C$ values we used the FA internal standard FREE 23:0 which was added before total lipid extraction in every sample. Altogether, the $\delta^{13}C$ value of 13 FAs was obtained (14:0, 15:0, 16:0, 16:1ω-7c, 18:0, 18:1ω-9, 18:1ω-7, 18:3ω-6, 18:3ω-3, 18:4ω-3, 20:4ω-6, 20:5ω-3, 22:6ω-3).

3.7 Data analysis

Analysis of variance (ANOVA) was used to test the effects of DOC concentration on the growth (specific growth rate) and FA content per cell of *Cryptomonas* sp. (I). If a significant ANOVA result was obtained, multiple comparisons between the phototrophic control and the rest of the treatments were carried out with Dunnett's test after verifying for homogeneity of variances (Levene's test). In addition to these results, IsoError version 1.04 (Phillips and Gregg 2001) was used to perform a two-source carbon isotope mixing model to quantify the contribution of inorganic (^{13}C-enriched) and organic carbon (DOC mix) integrated into phospholipid-rich fractions (I). This analysis was carried out only in DOC treatments, hence DOC1.5 was selected as the purely phototrophic condition due to its negligible light limitation. The average contribution of phototrophy (*fA*) was calculated as follows:

$$fA = \frac{(\delta M - \delta B)}{(\delta A - \delta B)},$$

where δM is the $\delta^{13}C$ value of DOC treatment been analysed (10, 30, 50 and 90 mg C l^{-1}), δA is the $\delta^{13}C$ value of DOC 1.5, and δB is the $\delta^{13}C$ value of DOC mix. Variation of $\delta^{13}C$ values of the two sources varied more than 4 %, therefore isotopic fractionation was not considered in the model.

When studying the interacting effects of eutrophication and warming on ten species of phytoplankton (II), PUFA proportion was first calculated by dividing total PUFA content by the sum of mono- and saturated fatty acid contents. Then, daily gain (µg FA l^{-1} d^{-1}) was used as a proxy for FA production (II) and its calculation was performed as follows:

$$\text{Daily gain} = FA_{cell}\ x\ \frac{(Cell_f - Cell_i)}{days}\ x\ \frac{1}{10^6},$$

where FA_{cell} is the FA concentration per cell (fg $cell^{-1}$) measured during the last day of cultivation, $Cell_f$ is the cell density (cells ml^{-1}) during the last day of cultivation, $Cell_i$ is the cell density (cells ml^{-1}) during the first day of cultivation, $days$ is the number of days elapsed between $Cell_f$ and $Cell_i$.

ANOVA was also used to study the effects of phosphorus (eutrophication) and temperature (warming) in ten phytoplankton (II) after verifying for homogeneity of variances (Bartlett's test). The cultures of the golden algae species *Synura* sp. rapidly collapsed under low phosphorus (LP) concentrations, hence only high phosphorus (HP) treatments were studied and statistically compared (II). If a significant ANOVA result was obtained from the individual species analysis (II), pairwise comparisons between treatments were conducted with Tukey's honestly significant difference (HSD) test. If data presented a significant Bartlett's test result (indicating unequal variances), Kruskal-Wallis rank sum test was performed to assess the effects of the studied treatments. Non-parametric pairwise comparisons were also carried out with Kruskal-Wallis test using Bonferroni correction. Glass' Δ (Lin and Aloe 2020) was used to study the effect size of increasing temperature and phosphorus (II). This estimate standardizes the difference in mean values between a control group and a test group by taking into account the standard deviation observed in the control group. These values are presented as heatmaps, where for each comparison between two treatments, the first treatment is considered as the control group for calculating Glass' Δ.

To calculate FA-specific production values (III), the FA $\delta^{13}C$ values obtained from both ^{13}C-enriched and natural seston samples were first converted to the abundance ratio of the heavy isotope (^{13}F) using the formula developed by Fry (2006):

$$^{13}F = \frac{(\delta^{13}C_{FA} + 1000)}{(\delta^{13}C_{FA} + 1000 + 1000/R_s)},$$

where $\delta^{13}C_{FA}$ is the δ^{13}C value of each analysed FA, and R_s is the Vienna Pee Dee Belemnite (VPDB) standard $^{13}C/^{12}C$ value = 0.01118 (Ding *et al.* 2001). To distinguish between volumetric and per content FA production, two units of production were proposed based on our data (III). The first unit, defined as volumetric production, refers to the production of phytoplankton FAs per litre per hour. The second unit, defined as productivity, refers to FA production per seston dry weight per hour. The volumetric production and productivity of each FA were calculated as follows:

$$\text{Production}_{\text{FA}} \text{or Productivity}_{\text{FA}} = \frac{(^{13}\text{F}_\text{E} - {}^{13}\text{F}_\text{N}) \times \text{C}_\text{FA}}{Time_{inc}} \; x\,1000\,,$$

where $^{13}F_E$ and $^{13}F_N$ are the abundance ratios of the studied FA in the ^{13}C-enriched and natural samples, respectively. C_{FA} is the carbon concentration or content (for the calculation of production or productivity, respectively) of the studied FA in the ^{13}C-enriched sample, and $Time_{inc}$ is the incubation time. Volumetric production was obtained by using a volumetric C_FA (ng l^{-1}), while productivity used a content C_FA (ng mg^{-1}). Total volumetric production (TVP) and total productivity were calculated as the sum of all calculated individual FA productions. For the analysis of relationships between TP and production, linear, logarithmic (where one or both the predictor and response variable are transformed using natural logarithm), and non-linear models were fitted (III). Model selection was based on Akaike Information Criterion (AIC). Models presenting a clear trend in their residual plot (not randomly distributed) were automatically excluded from further analysis. For non-linear models, non-parametric bootstrapping was used to obtain 95 % confidence intervals of model coefficients. Rejection of null hypothesis of non-linear model coefficients was done by looking at p-values, confidence intervals, and normality (visual checking) of frequency distribution of coefficient values obtained by nonparametric bootstrapping (1000 iterations). If frequency distribution was classified as skewed, the null hypothesis was not rejected due to the uncertainty in the confidence interval of the model parameter. The tested non-linear models correspond to density-independent Beverton and Holt (1957) (herein referred as Beverton-Holt model) and density-dependent Ricker (1954) models. In the density independent Beverton-Holt model the response variable approaches an asymptotic value as the independent variable increases. The density-dependent Ricker model describes a response variable that decreases beyond a threshold in the independent variable. Formula of the non-linear Beverton-Holt (1957) model is defined as follows:

$$y = \frac{ax}{(1+b)},$$

where y is the response variable, x the independent variable, a is the density-independent parameter, and b is the density-dependent parameter. The other candidate model by Ricker (1954) is defined as follows:

$$y = ax\, e^{\left(\frac{-ax}{Pp\, e}\right)} \quad ,$$

where y is the response variable, x is the independent variable, a is the density-independent parameter, Pp is the peak value for y, and e is Euler's constant. The same described linear and non-linear models were studied for the relationship between EPA and DHA contents of zooplankton and fish with TP. By doing so, the objective was to identify similarities between the trends observed in phytoplankton production of EPA and DHA and the contents of these PUFAs in higher trophic levels. Biomass of phytoplankton and zooplankton, and fish relative biomass (biomass per unit of effort) change with increases in TP was studied testing linear and logarithmic models. Model selection was also based on AIC values, and the same criteria (distribution of residuals) was used to discard models that did not described well the mean structure of the data. The limit of statistical significance was set to:

$$\alpha = 0.05 \,,$$

in all analyses (I, II, and III). All modelling and statistical tests were carried out using R (R Core Team 2019).

4 RESULTS AND DISCUSSION

4.1 Browning and phytoplankton mixotrophy

The growth of the studied mixotrophic cryptophyte was not impaired by light attenuation caused by browning (I, Fig. 2). On the contrary, DOC concentrations of 10 and 90 mg C l^{-1} were able to enhance the growth of *Cryptomonas* sp. over phototrophic conditions in a comparable manner to glucose supplementation (mixotrophy via osmotrophy). These results align with previous findings that mixotrophic phytoplankton dominate communities in high DOC boreal lakes (Bergström *et al.* 2003, Deininger *et al.* 2017). The competitive advantage that mixotrophy provides over obligated phototrophy helps explain why nitrogen fertilization of high DOC lakes (~20 mg C l^{-1}) increased the biomass of already established mixotrophic species but had little effect on phytoplankton communities (Deininger *et al.* 2017). Nevertheless, browning has been shown to decrease phytoplankton biomass at DOC concentrations > 11 mg C l^{-1} despite nutrient fertilization (Bergström and Karlsson 2019), indicating that the higher growth of mixotrophic phytoplankton cannot compensate for the decrease in obligated phototrophic biomass.

Despite light playing a role in controlling FA distribution and content in photosynthetic organisms (Valentine and Valentine 2004, Wacker *et al.* 2016), browning did not alter *Cryptomonas* sp. PUFA contribution or content per cell (Fig. 2). These results suggest that the quality of phytoplankton for herbivorous zooplankton is not altered by mixotrophic growth. Although we did not statistically test for differences in EPA and DHA contents individually, PUFA contribution and content results suggest that it is unlikely that big differences in the content per cell of these high quality PUFAs would have been observed with browning. Therefore, it seems unlikely that the overall quality of phytoplankton

biomass in lakes affected by browning will decrease as a consequence of a shift from phototrophic to mixotrophic phytoplankton communities.

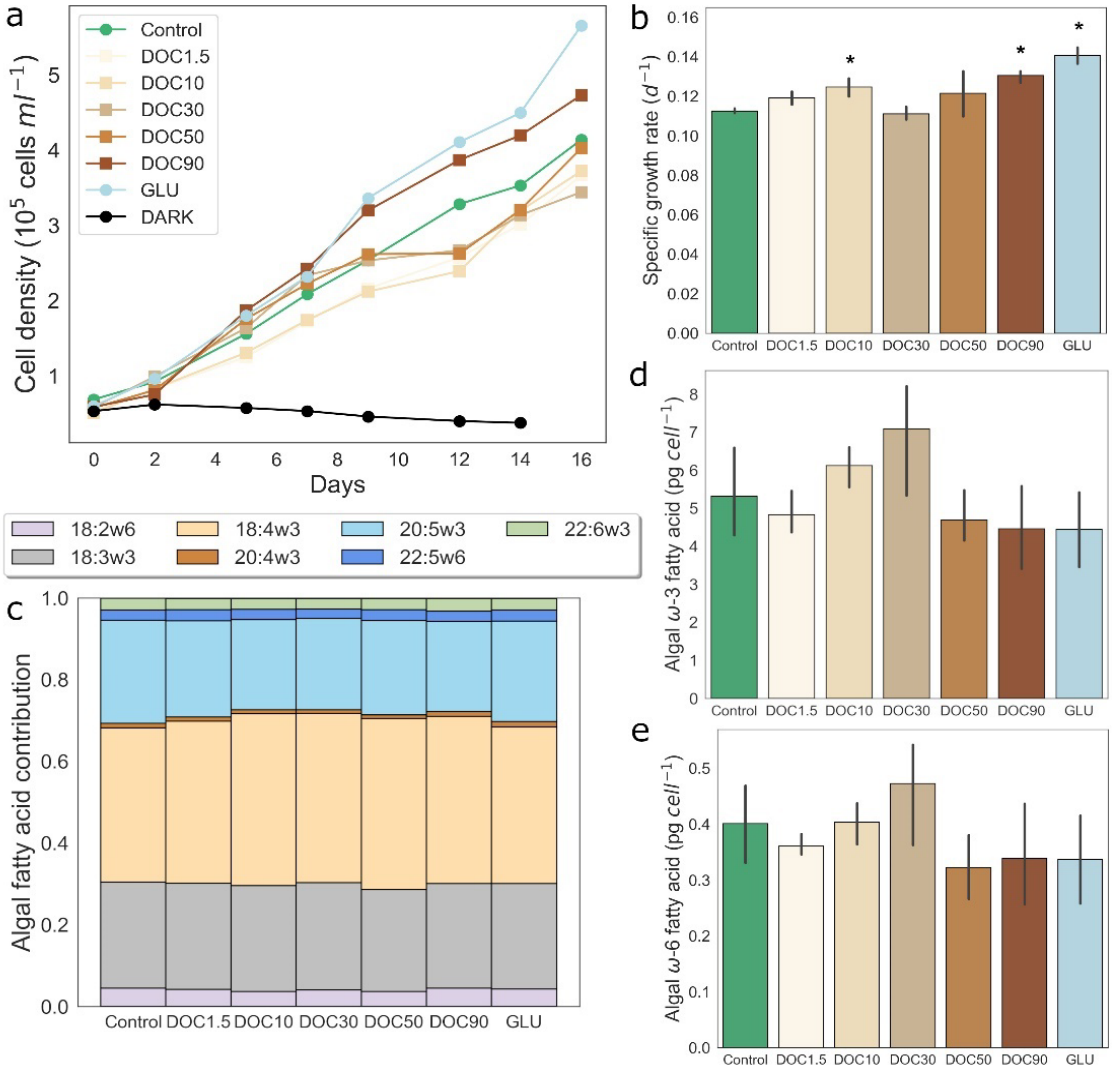

FIGURE 2 Growth and fatty acid (FA) responses of the mixotrophic cryptophyte *Cryptomonas* sp. to increases in DOC concentrations as compared to control conditions. DOC concentrations ranged from 1.5 to 90 mg C l^{-1} while controls represent phototrophic (Control), glucose-supplemented phototrophic (GLU), and heterotrophic (DARK) conditions. Cell density (a), calculated growth rates (b), algal fatty acid contributions (c), and total algal ω-3 and ω-6 fatty acid per cell (d and e, respectively) are shown in the figure. Values represent the mean and standard deviation of three to four replicates for b, d, and e, while only the mean is given in a and c. An asterisk (*) is used to denote a statistically significant difference between the treatment and control. Adapted from I, Figures 1, 2.

Cryptomonas sp. primary production decreased along the DOC gradient, with heterotrophically-acquired carbon becoming more relevant to sustain growth (Fig. 3). Since growth rates were equal or higher in the studied DOC concentrations than under phototrophic conditions, these results suggest that

organic carbon fully compensates the decrease in the fixation of inorganic carbon to produce biomass. In boreal lakes, primary production has been shown to decrease with DOC concentrations > 5 mg C l^{-1} (Seekell *et al*. 2015). Based on our results, mixotrophic phytoplankton growth becomes more reliant on organic carbon with decreasing light availability, lowering the fixation of inorganic carbon but maintaining biomass. Nevertheless, it is also possible that the decrease in primary production observed with browning is partially explained by the decrease in obligated phototrophic phytoplankton contribution to primary production.

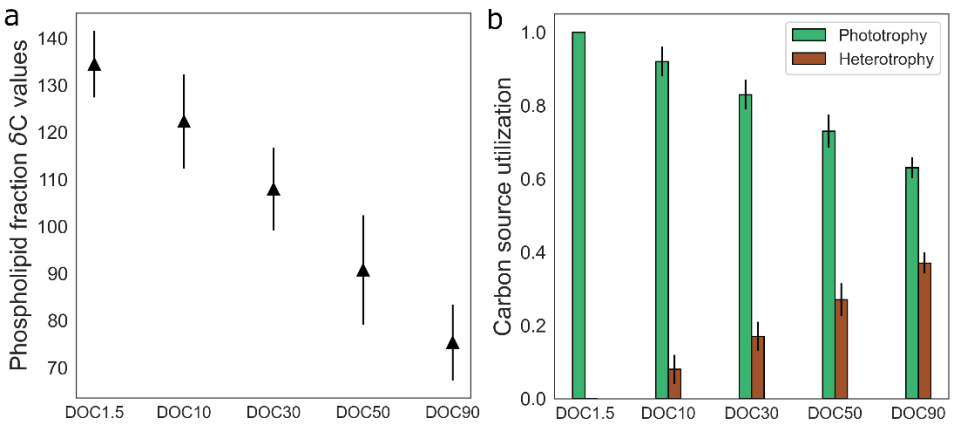

FIGURE 3 Effect of browning on inorganic (phototrophy) and organic (heterotrophy) carbon utilization. Phospholipid fraction δC analysis (a) of 5 % ^{13}C-$NaHCO_3$ supplemented DOC treatments. Two-source mixing model (b) results using DOC 1.5 as completely phototrophic. Values represent the mean and standard deviation of three to four replicates (I).

Osmotrophy and phagotrophy are the two mechanisms used by mixotrophic phytoplankton to obtain organic carbon (Flynn and Butler 1986, Flynn *et al.* 2012, Stoecker *et al.* 2017). Compared to phototrophic conditions (control), *Cryptomonas* sp. expression of phagotrophy-related genes (lysosome, phagosome, and endocytosis) tricarboxylic acid cycle genes increased along the DOC gradient (Fig. 4). These results suggest that bacteria-derived carbon sustains the mixotrophic growth of *Cryptomonas* sp. with browning. Additionally, browning led to lower expression of photosynthesis-related genes compared to control, validating the lower observed inorganic carbon fixation along the studied DOC gradient. Interestingly, the lowest tested DOC concentration (1.5 mg C l^{-1}) already presented overexpression of phagotrophy related genes and down-regulation of photosynthesis compared to control. This suggests that the transition to mixotrophic growth occurs even when light is not restricted, possibly to minimize the energetic expenditure associated with maintaining the photosynthetic apparatus (Raven 1997). Increased phagotrophy with browning align with prior observations made in high DOC lakes, where increases in bacterial biomass were rapidly followed by mixotrophic phytoplankton blooms (Bergström *et al.* 2003). Therefore, decreases in light availability may be compensated by increases in DOC and bacteria biomass in lakes dominated by

mixotrophic phytoplankton. Considering future predicted changes impacting boreal lakes, the presented results support previous projections (Hessen 1998, Creed *et al.* 2018, Blanchet *et al.* 2022) that browning will decrease phytoplankton primary production and increase reliance of food webs in bacterial biomass.

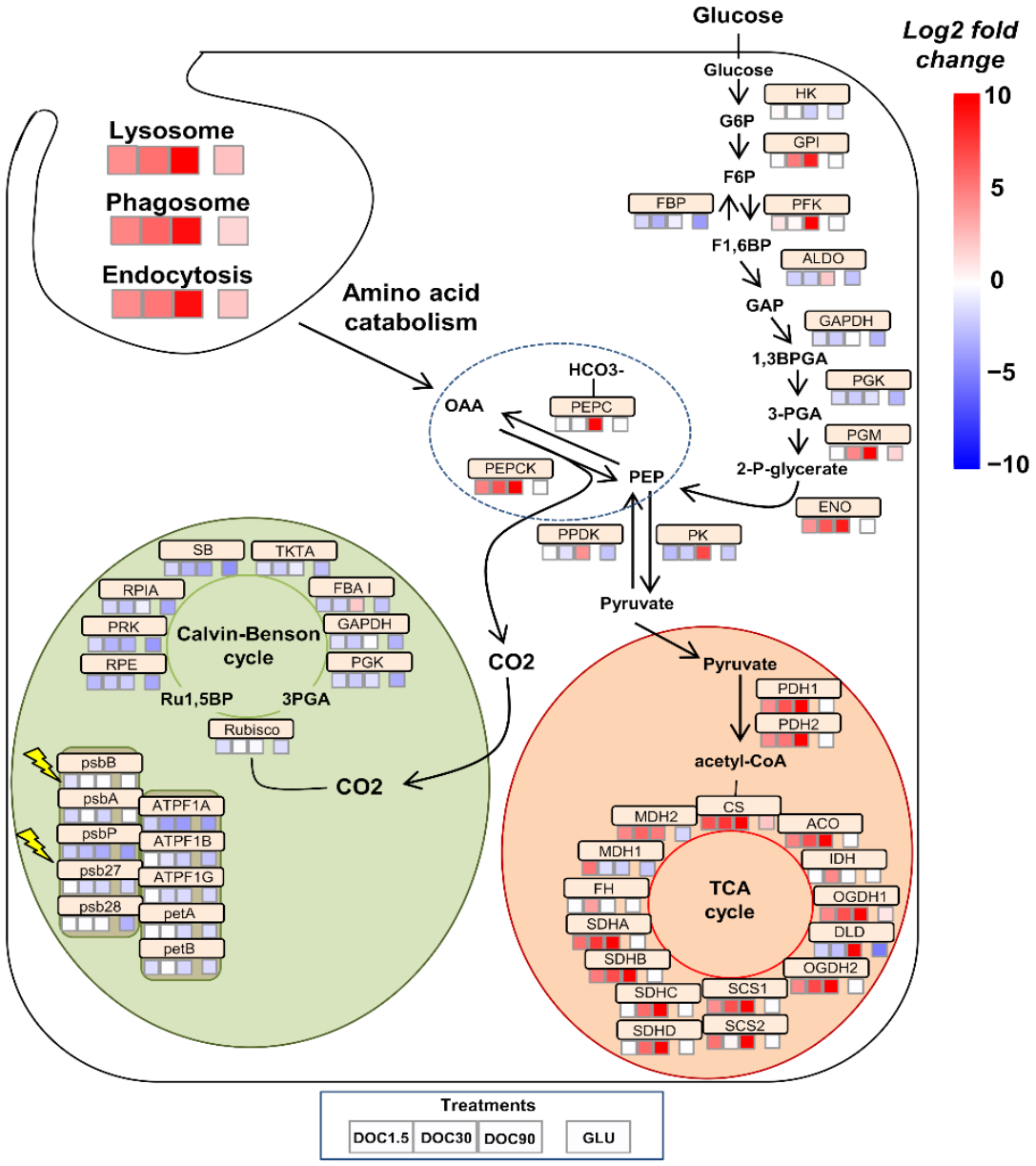

FIGURE 4 Changes in expression of genes related to photosynthesis, glycolysis and tricarboxylic acid cycle (TAC) with browning (DOC1.5, 30, and 90 mg C l^{-1}) and glucose supplementation (GLU). Log_2 fold-change values of the change in expression between the referred treatment and phototrophic control are indicated in squares beneath gene names. For the case of gene groups related to phagotrophy (lysosome, phagosome and endocytosis), squares represent the average Log_2 fold-change value of all up-regulated genes in that category. Green is used to represent plastids, red mitochondrion, and white cytosol. Values represent the mean and standard deviation of three replicates (I).

In addition, since browning favours mixotrophy (via phagotrophy) over phototrophy, this constrains the number of phytoplankton species that can grow efficiently under browning conditions. Consequently, browning is likely to decrease the diversity of phytoplankton as already observed in high DOC lakes (Jones 1992, Bergström *et al.* 2003, Deininger *et al.* 2017). Nevertheless, in terms of the quality of production, we did not observe any impact to PUFA contribution or content. Moreover, cryptophytes (particularly the genus cryptomonas) are high quality food sources for zooplankton (Peltomaa *et al.* 2017). Contrary to previous hypothesis (Hessen 1998, Creed *et al.* 2018), quality of phytoplankton production and energy transfer efficiency (phytoplankton to zooplankton) in browning lakes could potentially be improved by shifting from phototrophic to mixotrophic dominated communities. However, the overall reduction in phytoplankton biomass observed with browning under natural conditions (Bergström and Karlsson 2019) will restrict the total amount of energy available for higher trophic levels.

4.2 Warming and eutrophication effect on phytoplankton growth and quality

Both warming and higher phosphorus availability positively impacted the growth of ten boreal phytoplankton species from six phylogenetically distant groups (II, Fig. 5). These results were not surprising since warming and eutrophication driven by phosphorus boost phytoplankton growth under natural conditions (Hasler 1947, Schindler 1977, Hood *et al.* 2018, Keva *et al.* 2020). At the species level, differences in the effect size of increasing temperature and phosphorus were noticeable, with the cyanobacteria *Microcystis* sp. been strongly benefited by all studied combinations of environmental change. Species-specific cyanobacteria blooms in warm and nutrient-rich freshwater and coastal ecosystems are common (O'Neil *et al.* 2012, Vuorio *et al.* 2020a, Dai *et al.* 2023), suggesting that certain cyanobacteria species will become more prominent with warming and eutrophication. In addition, higher overall cyanobacterial contributions to phytoplankton communities are observed in eutrophic lakes (Taipale *et al.* 2016, Keva *et al.* 2020), strongly suggesting that intensified eutrophication and warming of boreal lakes will increase the share of cyanobacteria in phytoplankton communities.

Climate-driven warming of lakes is expected to decrease the availability of PUFAs in phytoplankton due to the fluidizing effect of temperature on cellular membranes (Sinensky 1974, Hixson and Arts 2016, Colombo *et al.* 2019). However, higher phosphorus availability promoted an increase in PUFA proportion in the studied phytoplankton species, suggesting that eutrophication could counterbalance the effect of temperature. The variability in species responses to environmental change is highlighted by the observed differences in the effect size of temperature and phosphorus. The underlying mechanism

responsible for these differences between species remains unclear, although we hypothesized that species-specific changes in membrane lipid classes driven by phosphorus limited conditions may be a contributing factor (Van Mooy *et al*. 2009, Matsui *et al*. 2020).

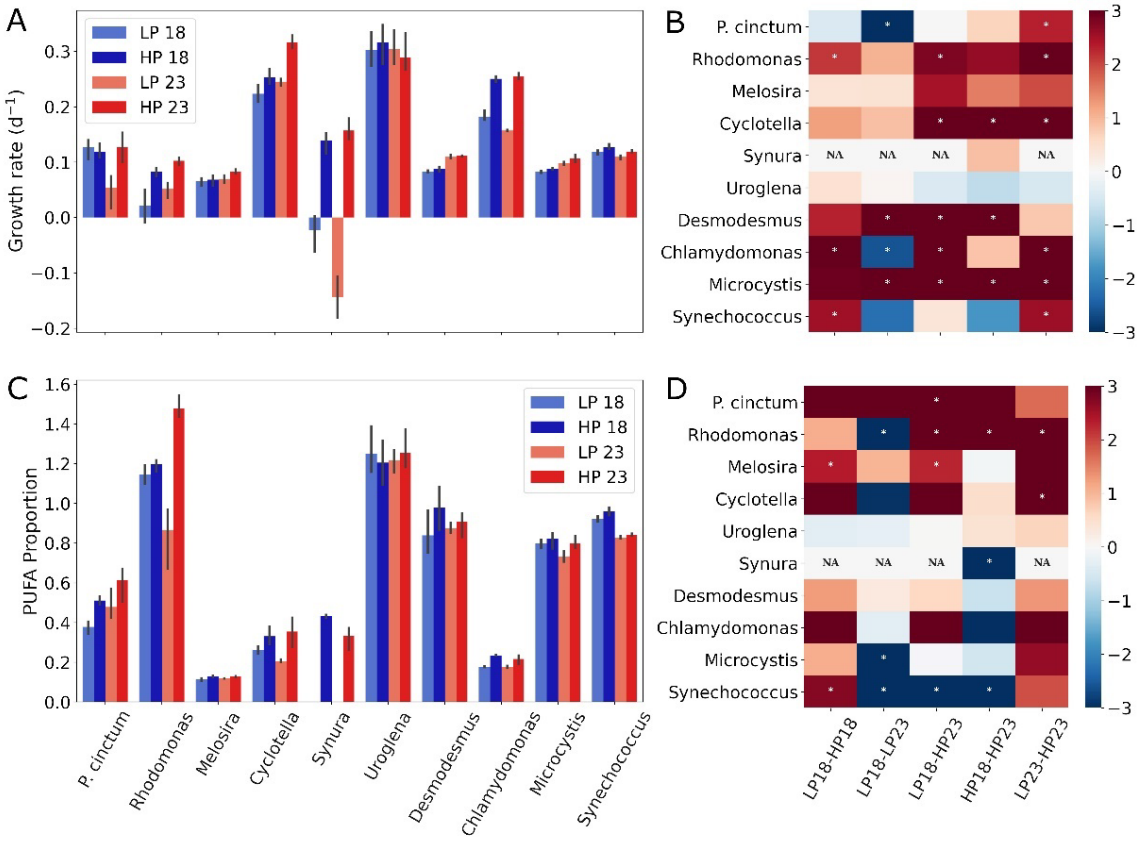

FIGURE 5 Growth rate (A) and polyunsaturated (PUFA) to mono- and saturated fatty acids proportion (C) changes to alterations in temperature and phosphorus. Size effect (Glass' Δ) of increases in temperature and phosphorus using the first referred treatment as baseline are shown for growth rate (B) and PUFA proportion (D). Phytoplankton species are representatives of the groups diatoms (*Cyclotella* sp. and *Melosira* sp.), golden algae (*Synura* sp. and *Uroglena* sp.), cyanobacteria (*Microcystis* sp. and *Synechococcus* sp.), green algae (*Chlamydomonas reinhardtii* and *Desmodesmus maximus*), cryptophytes (*Rhodomonas* sp.), and dinoflagellates (*Peridinium cinctum*). Treatment names designate culture conditions where 18 and 23 are the culture temperatures in °C and LP and HP refer to phosphorus concentration (0.65 [LP] and 2.58 [HP] µM phosphorus). An asterisk (*) is used to denote a statistically significant difference between the treatment comparisons for each phytoplankton species. Adapted from II, Figures 1, 3.

The effect of eutrophication and warming on production of EPA and DHA, accounted as FA daily gain, presented large variations in both directionality and size effect between species (Fig. 6). In addition, variation in magnitude of production between the studied species highlights the asymmetric role of different phytoplankton groups in the supply of EPA and DHA to food webs. Our results agree with previous studies where diatoms are recognized as key EPA producers, whereas DHA production is linked with dinoflagellates

(Ahlgren *et al.* 1992, Galloway and Winder 2015, Taipale *et al.* 2016). Overall, only DHA production was on average negatively impacted by warming, although this effect was less clear with concomitant eutrophication. The observed production responses to higher temperature and phosphorus challenge current forecasts of a substantial decline in phytoplankton EPA and DHA due to warming (Hixson and Arts 2016, Colombo *et al.* 2019). Furthermore, production differences between species and species-specific responses to environmental change emphasize that alterations in phytoplankton communities exert a significant influence on the availability of high-quality PUFAs.

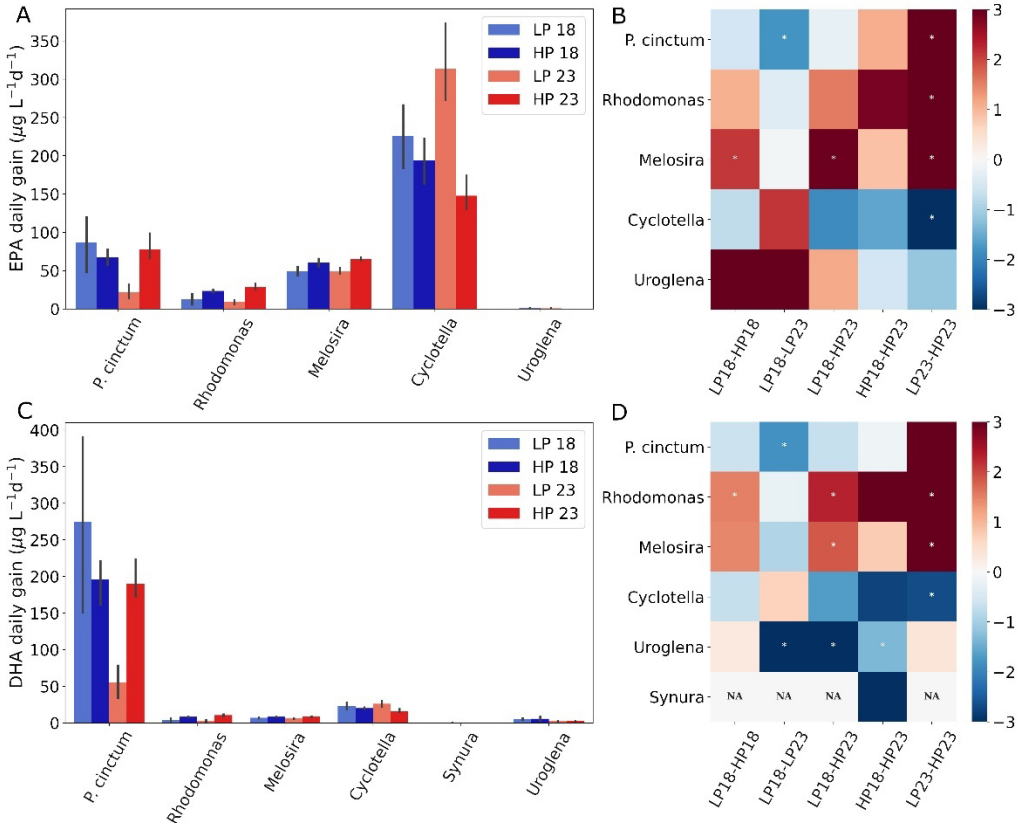

FIGURE 6 Changes in EPA (A) and DHA (C) production (calculated as daily gain) with variations in temperature and phosphorus concentration. Size effect (Glass' Δ) of increases in temperature and phosphorus using the first referred treatment as baseline are shown for EPA (B) and DHA (D) production. Phytoplankton species are representatives of the groups diatoms (*Cyclotella* sp. and *Melosira* sp.), golden algae (*Synura* sp. and *Uroglena* sp.), cryptophytes (*Rhodomonas* sp.), and dinoflagellates (*Peridinium cinctum*). Treatment names designate culture conditions where 18 and 23 are the culture temperatures in °C and LP and HP refer to phosphorus concentration (0.65 [LP] and 2.58 [HP] µM phosphorus). An asterisk (*) is used to denote a statistically significant difference between the treatment comparisons for each phytoplankton species (II).

Importantly, laboratory studies looking at the effect of temperature on PUFA availability focused on content analysis (FA per mg of dry weight) (Hixson and Arts 2016, Colombo *et al.* 2019). The limitation of such measurement is that it does not account for phytoplankton growth when estimating PUFA availability for higher trophic levels. Lower phytoplankton contents of high nutritional value

FAs such as EPA and DHA can be compensated by higher growth, stabilizing their supply for higher trophic levels. For the future of boreal lakes, the obtained results suggest that higher phosphorus inflow from catchments could counteract the effect of warming on the quality of production per species. In addition, it is also possible that lakes of different trophic status will respond differently to warming in terms of their quality of production. Overall, future availability of EPA and DHA will strongly depend on community composition, with diatoms and dinoflagellates playing a crucial role in the availability of these PUFAs.

4.3 Eutrophication effects on quality of production and lake food web

Lakes in central and southern Finland along a eutrophication (TP and total nitrogen) gradient showed notable changes in food web community, biomass, phytoplankton production, and transfer of EPA and DHA (III). Phytoplankton communities displayed significant variability along the TP gradient (Fig. 7). However, as previously reported (Donald *et al.* 2011, Taipale *et al.* 2016, Keva *et al.* 2020), the net impact of eutrophication was an overall reduction of diatom abundance accompanied by an increase in cyanobacteria. Additionally, eutrophication increased the contribution of non-edible phytoplankton (≥ 35 um), which is not available for herbivorous zooplankton due to its large size (Porter 1973, Knisely and Geller 1986). This reduction in the share of edible phytoplankton may be attributed to higher zooplankton biomasses grazing on small, nutrient-rich phytoplankton cells (Meise *et al.* 1985, Meunier *et al.* 2015). As a result, large non-edible phytoplankton remains in the water column. When accounting for edibility, eutrophication did not greatly impact edible phytoplankton communities, with cryptophytes and dinoflagellates presenting high contributions across the TP gradient. Zooplankton communities were relatively stable along the eutrophication gradient, with most of the studied lakes been dominated by copepods. A noticeable trend was observed in fish, where eutrophication shifted communities from percid to cyprinid-dominated, driven by a decrease in perch and an increase in roach, common bream, and white bream contributions. Such changes in fish communities are congruent with previous observations (Persson 1991, Olin *et al.* 2002, Hayden *et al.* 2017), suggesting that cyprinids have a competitive advantage over percids in eutrophic environments. Biomass of phytoplankton and zooplankton, as well as relative fish biomass (biomass per unit of effort), increased in response to eutrophication (Fig. 7). These results are consistent with earlier findings supporting the positive influence of increased nutrient availability on the total biomass of food webs (Hasler 1947, Schindler 1977, Jeppesen *et al.* 2000, Keva *et al.* 2020). However, there were differences in the rate of biomass increase per unit of TP between trophic levels, with phytoplankton presenting more pronounced increases than zooplankton per increase in TP. These results agree with the 'Green World Hypothesis' where

phytoplankton are bottom-up controlled by nutrients and zooplankton are top-down controlled by fish (Hairston *et al.* 1960).

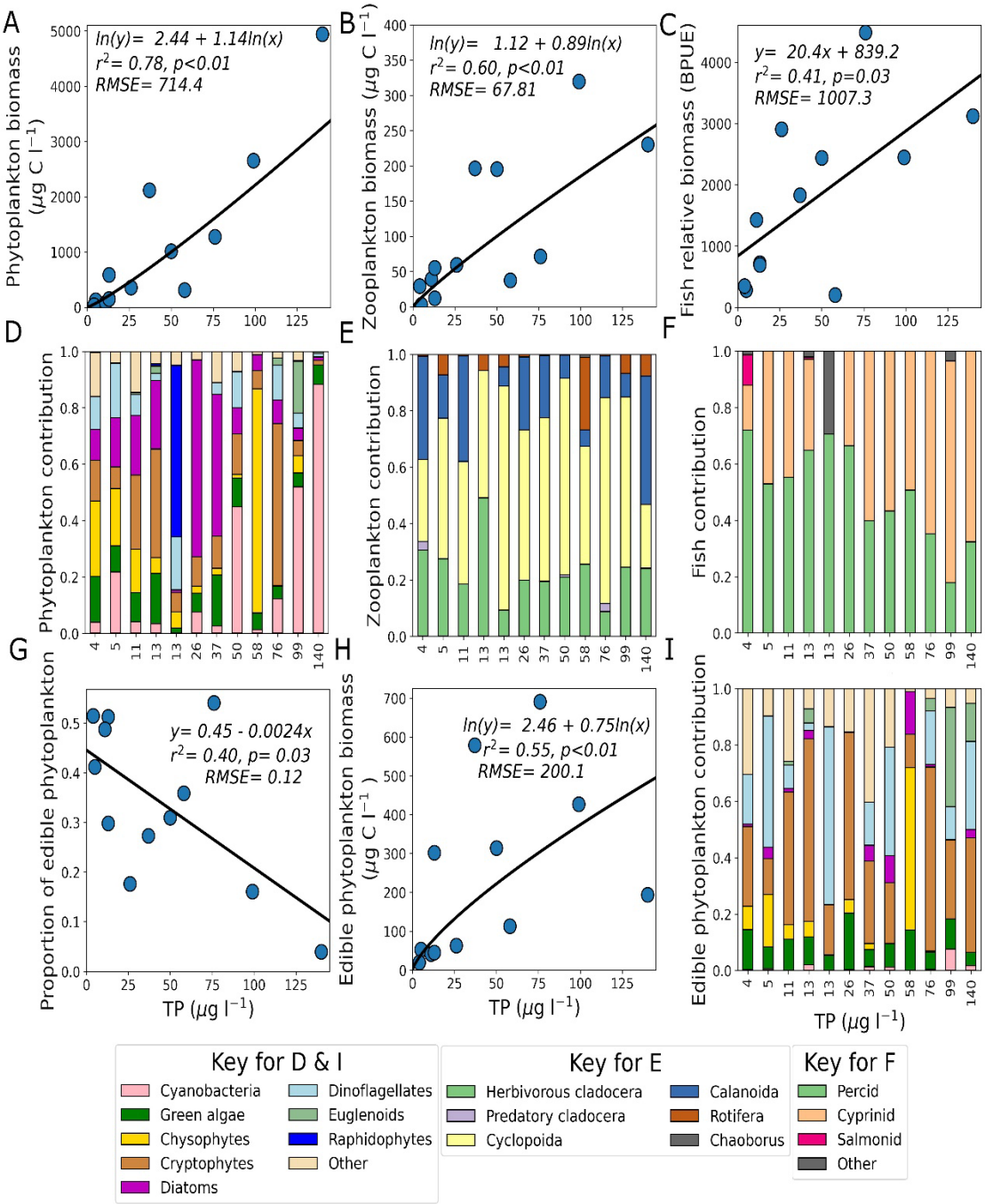

FIGURE 7 Biomass, relative biomass, and community composition of phytoplankton, zooplankton, and fish across a total phosphorus (TP) gradient (4–140 µg l^{-1}). Total and edible phytoplankton biomass (A and H, respectively), as well as their community composition (D and I, respectively) are presented. Phytoplankton was classified as edible based on size (< 35µm) and its proportional change with TP is presented in (G). Zooplankton biomass (B) and community composition (E) are presented. Fish relative biomass (C) and community composition (F) were obtained with biomass per unit of effort (BPUE) for each lake. Regression models (A, B, C, G, and H) display Root Mean Square Error (RMSE), p-value (p) and coefficient of determination (R^2) (III).

Volumetric production and productivity, which are proposed units to study phytoplankton primary production, exhibited distinctive patterns with eutrophication (Fig. 8). Total volumetric production (TVP) was best described by a saturating (Beverton-Holt equation) relationship with eutrophication, while total productivity showed a unimodal (Ricker function) response to this increase in nutrients. In oligotrophic lakes, nutrient increases stimulated both TVP and total productivity, likely by an increase in both phytoplankton biomass and photosynthetic fixation of inorganic carbon per cell (Zurano *et al.* 2021). Since TVP depends on phytoplankton biomass, the relationship between these variables was studied to account for the variability observed in TVP within eutrophic lakes (TP > 35 µg l^{-1}). A saturation non-linear relationship, modelled via the Beverton-Holt equation, provides the best description of the changes in TVP per unit of phytoplankton biomass.

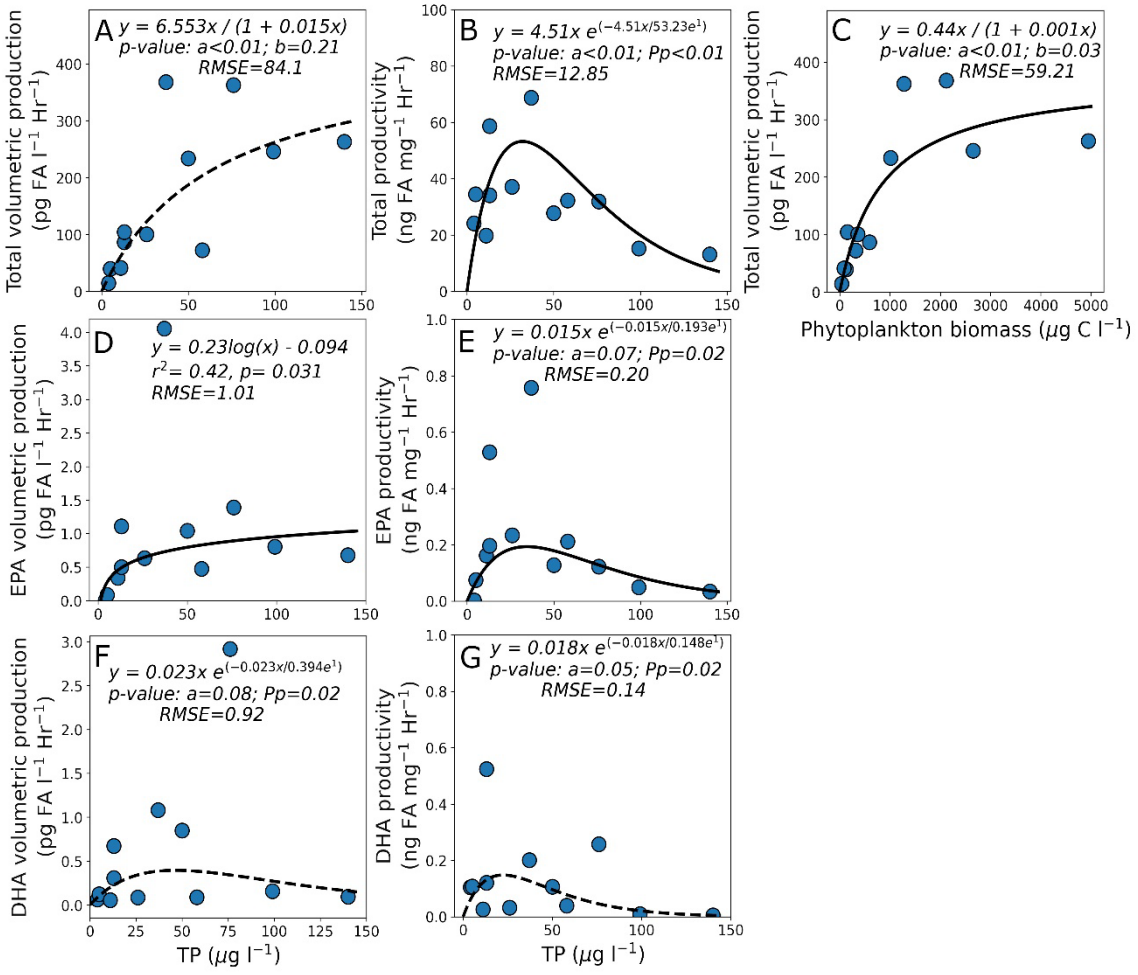

FIGURE 8 Eutrophication effect on total, EPA, and DHA volumetric production (A, D, and F, respectively) and productivity (B, E, and G, respectively). Additionally, the effect of increasing phytoplankton biomass on total volumetric production (C) is presented. Unimodal relationships (B, E, F, and G) were modelled with a Ricker equation while saturating models (A and C) were modelled with a Beverton-Holt equation. Dashed lines indicate nonsignificant models ($p > 0.05$). All models display Root Mean Square Error (RMSE) while only logarithmic model (D) present their coefficient of determination (R^2) (III).

Considering the negative effect of eutrophication in total productivity past the range of TP that provides maximal productivity (95 % confidence interval: 28–39 μg l^{-1} TP), our results suggest that when phytoplankton densities are higher than the biomass required to achieve half the TVP saturating concentration (> 850 μg C l^{-1}), competition for nutrients and cell shading decrease phytoplankton photosynthetic capacity (Han *et al.* 2000, Day *et al.* 2012). These results agree with the "Paradox of enrichment", where at high nutrient availability, further increases in nutrients reduce productivity (Rosenzweig 1971). Taken together, these results suggest that lakes with TP concentrations in the range of maximal productivity have the highest phytoplankton efficiency for fixation of inorganic carbon. In terms of quality of production, EPA volumetric production responded logarithmically to increases in TP, while a unimodal (best described by a Ricker equation) response was observed for EPA productivity. The TP range of maximal EPA productivity corresponded to 22–61 μg l^{-1} (95 % confidence intervals). DHA volumetric production and productivity were best described by a unimodal (Ricker) relationship with TP. Nevertheless, given the uncertainty in model parameters (skewed bootstrapping frequency plots) the null hypothesis cannot be rejected. The existence of a range of TP that maximizes EPA and DHA productivity is supported by previous estimations of EPA and DHA availability in lakes of different nutrient categories based on phytoplankton communities (Taipale *et al.* 2016). Altogether, the unimodal relationships observed in total and EPA productivity, and well as the best fitting model for DHA productivity, suggest that there is a range of TP where fatty acid synthesis per phytoplankton biomass is maximal, both in terms of quantity and quality. Across the studied lakes, the highest EPA volumetric production was observed in a diatom dominated lake (TP = 37 μg l^{-1}) with high phytoplankton biomass, highlighting the relationship between phytoplankton community and EPA production. Nevertheless, in lakes that are not dominated by a single phytoplankton group or have low total biomass, the correlation between phytoplankton community composition and EPA production is not so clear, likely because of other environmental conditions affecting EPA production. The lack of statistically significant trends in DHA volumetric production and productivity in the studied lakes suggest that other environmental variables besides nutrients modulate DHA production.

Comparison of EPA and DHA productivity to zooplankton and fish contents of these PUFAs showed no clear correlation (Fig. 9). Herbivorous cladocerans EPA and calanoids DHA contents showed a notable decrease with increasing TP, while no trend was observed for predatory cladocerans. Changes in cyclopoids DHA content with increases in TP was best described by a unimodal (Ricker) model. Based on this model, the TP range of maximal DHA content was 26–47 μg l^{-1} (95 % confidence interval). Interestingly, highest EPA and DHA contents in herbivorous cladocerans and calanoids (respectively) were observed in oligotrophic lakes, where both volumetric production and productivity of these PUFAs were low. Given that FA transfer between phytoplankton and zooplankton is thought to be linear (Dalsgaard *et al.* 2003,

Iverson 2009), the observed mismatch between EPA and DHA productivity and cladocerans and calanoids contents can be potentially explained by targeted grazing of high-quality phytoplankton in oligotrophic lakes (Meise *et al.* 1985, Meunier *et al.* 2015). In addition, selection pressures in oligotrophic lakes could favour zooplankton species with higher bioconversion efficiency of dietary PUFAs (Boyen *et al.* 2023). With increasing TP, the decrease in zooplankton EPA and DHA contents do not match the productivity dynamics of these PUFAs. Among possible explanations, phytoplankton size (edibility) could play a role in constraining the transfer of PUFAs. Variability in FAs within species of the same phytoplankton group (Ahlgren *et al.* 1992, Lang *et al.* 2011) limits the accuracy of estimating available (edible) EPA and DHA based on species composition. Additionally, nutrient conditions affect phytoplankton PUFA production (II). An example of the effect of phytoplankton edibility on EPA transfer can be observed in our highest EPA productivity lake (Lohjanjärvi, TP = 37 µg l^{-1}) where most of the diatom biomass was classified as non-edible for zooplankton. Consequently, zooplankton from this lake did not present higher EPA contents compared to the rest of the studied lakes. In addition to edibility, variations in the duration of life cycles and lipid accumulation strategies of zooplankton (Hiltunen *et al.* 2016), as well as the distinct partitioning of fatty acids towards somatic growth, reproduction, or catabolism (Galloway and Budge 2020), may potentially account for the mismatch between phytoplankton production and zooplankton content of EPA and DHA.

In fish, eutrophication decreased the EPA and DHA contents of perch while roach and ruffe were completely unaffected by eutrophication. These results agree with previous studies where low quality of phytoplankton and zooplankton were efficiently mitigated in some fish species (Kainz *et al.* 2017, Keva *et al.* 2020, Taipale *et al.* 2022). Interestingly, the EPA and DHA contents of smelt (pelagic planktivorous fish) replicated the unimodal relationship between EPA and DHA productivity and TP, despite only cyclopoids presenting this trend among zooplankton. The variability observed in the EPA and DHA contents of different fish species highlights how variability in feeding strategies, length of life cycles, and possibly metabolic differences, influence the content of these PUFAs. Bioconversion of shorter PUFAs to DHA has been described in perch (Ishikawa *et al.* 2019) and is likely to be present in other fish species, suggesting that bioconversion could play a more significant role in eutrophic lakes than previously thought. Nevertheless, not all food web components were sampled in this study (i.e., periphyton, benthic and pelagic macroinvertebrates, and others) which could partially explain the observed results. Considering that boreal lakes are expected to receive larger nutrient imports due to increases in precipitations (de Wit *et al.* 2016, Ruosteenoja *et al.* 2016, Isles *et al.* 2023), our results indicate that total and quality of production will be differently affected depending on the lakes' current nutrient status. Oligotrophic and mesotrophic lakes will likely benefit from moderate eutrophication, increasing both their total and EPA (and likely DHA) volumetric production by stimulating phytoplankton biomass and photosynthetic efficiency per cell. Nevertheless, this increase in

production of PUFAs is unlikely to directly transfer to zooplankton and fish. In eutrophic lakes, increases in TP over the estimated range of maximal total productivity (> 40 μg l^{-1}) will likely decrease the FA productivity driven by changes in both phytoplankton communities and species-specific production (II). Despite that zooplankton will further decrease its EPA and DHA contents in eutrophic lakes, fish species with adaptations to high nutrient concentrations are likely to mitigate low quality of production.

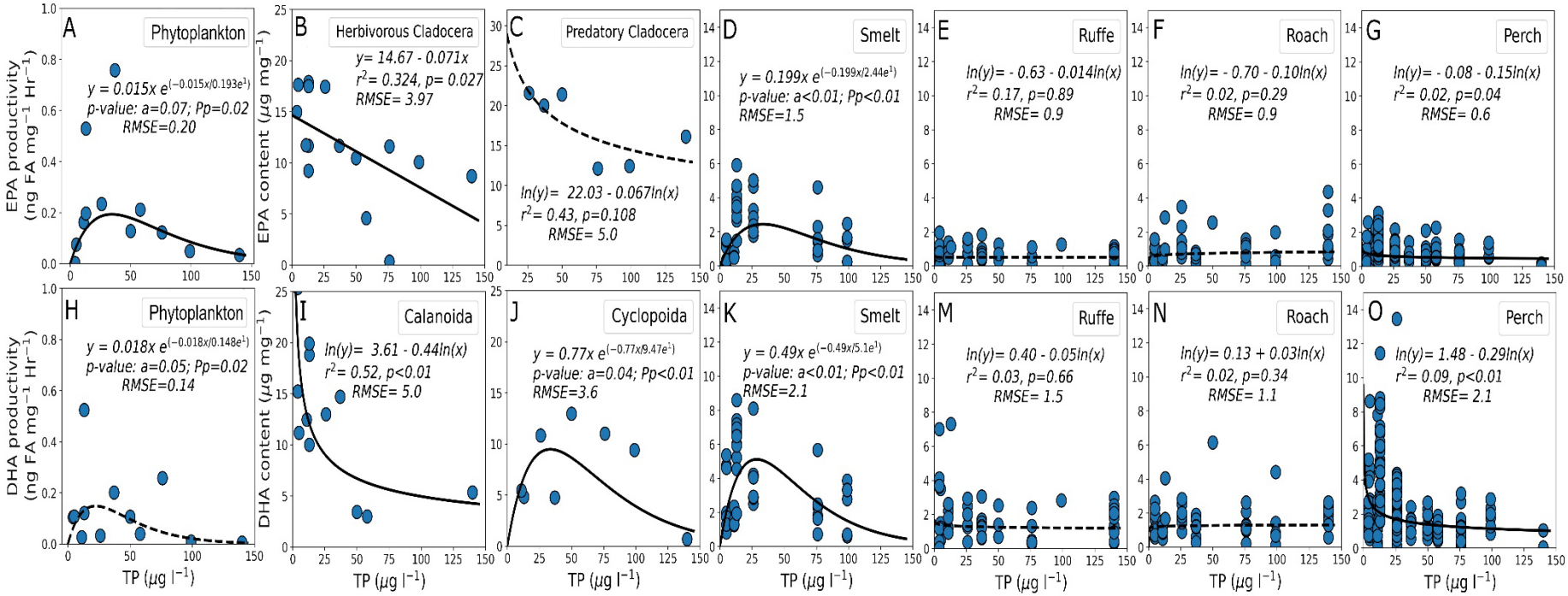

FIGURE 9 Eutrophication effect on EPA productivity (A) and content of herbivorous (B) and predatory Cladocera (C), smelt (D), ruffe (E), roach (F) and perch (G), as well as DHA productivity (H) and content in Calanoida (I), Cyclopoida (J), smelt (K), ruffe (M), roach (N) and perch (O). Dashed lines indicate nonsignificant models ($p > 0.05$). Density dependent non-linear models of the form Ricker (A, D, H, J, and K) present p-values obtained through parametric tests for each parameter. All models display Root Mean Square Error (RMSE) while only linear and logarithmic models present their coefficient of determination (R^2) (III).

4.4 Perspectives on the study of quality of production

Differences in methodology to calculate production, as well as the units in which production is evaluated, play a major role in the interpretation of results. As observed throughout the presented studies (I, II, and III), production of high nutritional quality PUFAs such as EPA and DHA can be approximated from incorporation of carbon into biomass (I), using FA daily gain (II), and with more advanced methods such as ^{13}C-labelling and CSIA (III). All these methods have pertinent applications for the study of production and transfer of FAs across food webs, but the nature of the measurement needs to be considered when interpreting the results. In the first study, conventional measurements of primary production (bulk incorporation of inorganic carbon into biomass) allowed to study how photosynthetic fixation of carbon varies with environmental change (I). Nevertheless, quality of production can be challenging to estimate with this technique given the effect of mixotrophy on fixation of inorganic carbon. In addition, under natural conditions different phytoplankton species contribute differently to primary production. Therefore, even with community composition data several assumptions should be made to correlate primary production and quality. Approximations like daily gain (II) can be used to study how changes in both cell number and FA content determine the true availability of PUFAs for higher trophic levels. It is important to consider that changes in cell growth can compensate for decreases in content (II), leading to different interpretations for natural conditions when only FA content is considered. Although this method is useful under laboratory conditions, direct application in natural conditions is limited due to the difficulty of accurately measure changes in cell densities. CSIA represents an alternative to conventional methods where production of individual FAs can be directly measured. Despite the usefulness of this methodology to study quality of production under natural conditions, the complexity inherent to aquatic food webs hinders CSIA potential to unravel the mysteries behind FA transfer (III). Zooplankton selection of prey, either by selective grazing or by constrains based on phytoplankton size, are a major challenge to understanding how FAs move from phytoplankton to zooplankton. Due to the variability in FA contents and photosynthetic fixation between phytoplankton species, it is not possible to discriminate what share of produced FAs is available to zooplankton. In addition, processes previously overlooked such as bioconversion or channelling of FAs towards catabolism could play a significant role in zooplankton. It is important to identify that only 12 lakes of different morphological characteristics were studied at one timepoint. Seasonality of environmental conditions, as well as differences in morphological characteristics, could play a role in the patterns of production and transfer of high-quality PUFAs.

5 CONCLUSIONS

Climate change and land use within catchment areas will affect the abiotic ecological boundaries of phytoplankton in boreal lakes. The altered environmental conditions will select for phytoplankton species with advantageous traits, affecting phytoplankton communities, biomass, and the quality of production. Mixotrophy confers an ecological advantage to phytoplankton under the low light conditions imposed by browning. This suggests that lakes submitted to intense browning will favour mixotrophic phytoplankton with the capacity to phagocytise bacteria to sustain growth. Importantly, the reduced fixation of inorganic carbon driven by higher mixotrophy will complicate measurements of total phytoplankton production and should be accounted for when studying high DOC lakes. Overall, cryptophytes (particularly cryptomonads) are high quality food sources for zooplankton, suggesting that the quality of phytoplankton biomass could increase in browning lakes dominated by low-quality phototrophic phytoplankton such as cyanobacteria. By stating this, I am not giving a quantitative estimate of how browning will affect quality of production, but establishing a conceptual model that helps understand the changes that browning lakes could face. In addition, it is important to highlight that only the response of one species was investigated, hence studies testing the same conditions in other mixotrophic species that are benefited by browning could elucidate more general patterns in phytoplankton.

The predicted warming-driven decreases in PUFAs across all species and nutrients levels were not observed across all studied phytoplankton species. Moreover, increases in phosphorus increased PUFA proportion despite higher temperatures. Production of the high quality PUFAs EPA and DHA showed no clear patters with warming and phosphorus across the studied species, limiting the use of environmental conditions to predict the availability of these PUFAs for higher trophic levels when no information of phytoplankton community composition is available. Furthermore, these results suggest that lakes of different trophic status could respond differently to warming. Therefore, I consider that a more cautious approach should be taken when making inferences about the future quality of production. Overall, I consider that higher temperature and nutrient availability will boost phytoplankton growth and select for species with adequate thermal niches and affinity for essential

nutrients. Phytoplankton responses to environmental change can vary substantially between species, highlighting the importance of community composition for the future quality of production in different aquatic ecosystems.

The changes observed in food web community and biomass of the 12 studied lakes across a nutrient gradient followed previously described patters. In terms of primary production, volumetric and per content production (productivity) showed non-linear relationships with TP. When accounting for changes in both volumetric production and productivity, the presented results point to a range of optimal TP where phytoplankton photosynthesis is very efficient, and communities have high production of EPA and DHA. The higher EPA and DHA productivity did not correlate with zooplankton and fish quality. I consider that other variables play a role in the transfer of FAs across trophic levels such as edibility of phytoplankton for zooplankton and metabolic processes related to EPA and DHA in consumers. It is important to point out that only 12 lakes at a single time point were sampled. A larger number of lakes of varying morphological characteristics, as well as measurements of FA production at different timepoints (seasonality of production), could fill the gaps between production and transfer observed. More datapoints of DHA production could potentially elucidate a pattern across nutrient conditions.

Future quality of production in boreal lakes will depend on the degree of browning, warming, and eutrophication that each lake is submitted to. These environmental changes will shape phytoplankton communities and total biomass, consequently impacting the availability of high quality PUFA such as EPA and DHA for higher trophic levels. Overall, I want to emphasize the technical difficulties and biological complexity involved in the study of phytoplankton quality of production and FA transfer across food webs. More research is required to understand phytoplankton FA responses to different types of environmental change. In addition, clarifying the biochemical mechanisms involved in the FA metabolism of zooplankton and fish, as well as zooplankton prey selection and genetic variability across environmental gradients is required. This information, together with estimations of size specific phytoplankton production obtained by ^{13}C-labelling of phytoplankton followed by CSIA, could be used to better understand the flow of EPA and DHA across aquatic food webs.

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ORIGINAL PAPERS

I

METABOLIC PLASTICITY OF MIXOTROPHIC ALGAE IS KEY FOR THEIR PERSISTENCE IN BROWNING ENVIRONMENTS

by

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ORIGINAL ARTICLE

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Metabolic plasticity of mixotrophic algae is key for their persistence in browning environments

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Abstract

Light availability is the main regulator of primary production, shaping photosynthetic communities and their production of ecologically important biomolecules. In freshwater ecosystems, increasing dissolved organic carbon (DOC) concentrations, commonly known as browning, leads to lower light availability and the proliferation of mixotrophic phytoplankton. Here, a mixotrophic algal species (*Cryptomonas* sp.) was grown under five increasing DOC concentrations to uncover the plastic responses behind the success of mixotrophs in browning environments and their effect in the availability of nutritionally important biomolecules. In addition to the browning treatments, phototrophic, heterotrophic and mixotrophic growth conditions were used as controls. Despite reduced light availability, browning did not impair algal growth compared to phototrophic conditions. Comparative transcriptomics showed that genes related to photosynthesis were down-regulated, whereas phagotrophy gene categories (phagosome, lysosome and endocytosis) were up-regulated along the browning gradient. Stable isotope analysis of phospholipid fractions validated these results, highlighting that the studied mixotroph increases its reliance on heterotrophic processes with browning. Metabolic pathway reconstruction using transcriptomic data suggests that organic carbon is acquired through phagotrophy and used to provide energy in conjunction with photosynthesis. Although metabolic responses to browning were observed, essential fatty acid content was similar between treatments while sterol content was slightly higher upon browning. Together, our results provide a mechanistic model of how a mixotrophic alga responds to browning and how such responses affect the availability of nutritionally essential biomolecules for higher trophic levels.

KEYWORDS
browning, *Cryptomonas*, dissolved organic carbon, fatty acids, mixotrophy, phagotrophy, transcriptomic

1 | INTRODUCTION

Primary production is centrally important to ecological processes since it provides energy and essential biomolecules for upper trophic levels. Ultimately, harvesting of light energy depends on light availability and the ability of phototrophic organisms to regulate photosynthesis according to environmental cues (Gerbaud & André, 1980). When light becomes limiting, the capacity of a photosynthetic organism to utilize alternative sources of energy can manifest in a competitive advantage over obligate phototrophs. Mixotrophy refers to the capacity of organisms to complement their photosynthetic activity with exogenous organic carbon sources (i.e., uptake of sugars, engulfment of bacteria) to maintain or enhance their fitness. While such metabolic flexibility can be advantageous with transient low light, energetic and nutrient investments in light harvesting machinery are high (Raven, 1997). Consequently, mixotrophs present plastic responses to changes in light and organic carbon availability to maximize fitness (González-Olalla et al., 2021). Such changes can be ecologically relevant since they potentially affect the availability of nutritionally important biomolecules for higher trophic levels (Brett et al., 2006; Peltomaa et al., 2017).

In freshwater and costal ecosystems, increases in terrestrial dissolved organic carbon (DOC) loading is a process commonly called browning. The term browning refers to the darkening of water towards a brown colour which leads to reduced penetration of shorter wavelengths of the visible spectrum of solar radiation (Graneli, 2012). In lakes, this reduction in light availability has been linked to reduced dissolved oxygen concentrations (Couture et al., 2015), and reduced primary production (Thrane et al., 2014), as well as higher costs of drinking water purification (Hongve et al., 2004). Given that browning is associated with reduced acid deposition, increased precipitation and increased terrestrial primary production in catchment areas (Kritzberg et al., 2020), it is expected that climate change will further intensify ongoing brownification processes in surface waters (Björnerås et al., 2017; Kritzberg & Ekström, 2012; Spilling et al., 2022). In particular, the boreal zone could suffer more intense browning given the combination of increased precipitation (12%–20% expected increase; IPCC, 2013; Ruosteenoja et al., 2016) and the predominance of water bodies with peatland-dominated catchments. In addition to changes in water colour, mobilization of terrestrial DOC is accompanied by macronutrients such as nitrogen and phosphorus and micronutrients such as iron (Ged & Boyer, 2013; Maranger & Pullin, 2003; Qualls & Richardson, 2003). Together, these modifications in the physical and chemical properties of water are expected to disturb the balance between algal and bacterial production (Creed et al., 2018), reduce energy transfer efficiency through food webs (Hessen, 1998), and affect the availability of essential fatty acids (FAs) and sterols for consumers (Martin-Creuzburg et al., 2009; Parrish, 2009; Peltomaa et al., 2017; Sargent et al., 1995).

Phytoplankton communities observed in highly brown environments (high DOC concentration) are less diverse (Jones, 1992) and are usually dominated by mixotrophic flagellated species (Bergström et al., 2003; Deininger et al., 2017; Wilken et al., 2018). Environmental data together with mesocosm studies have shown that mixotrophic cryptophytes, in particular the genus *Cryptomonas*, thrive in brown freshwater environments (Bergström et al., 2003; Deininger et al., 2017; Isaksson et al., 1999; Wilken et al., 2018), making them promising models of plastic and genetic adaptations to browning. Importantly, mesocosm studies suggest that mixotrophs respond to browning by enhancing bacterial predation (Wilken et al., 2018). Since bacteria make use of terrestrially derived DOC for growth (Kritzberg et al., 2006), and that active bacterial predation in mixotrophic algae has been linked to decreases in oxygen production (González-Olalla et al., 2021; Wilken et al., 2014), increasing algal mixotrophy with browning could alter the carbon and oxygen cycling of lakes. In addition, the trophic transition from phototrophic to mixotrophic, as well as variations in the physicochemical properties of water, can affect the FA and sterol content of algae (Boëchat et al., 2007; Peltomaa & Taipale, 2020; Piepho et al., 2010). Nevertheless, other responses to low light (i.e., changes in pigments), together with diel movements across the water column to avoid light limitation (Gervais, 1997), could also explain the success of mixotrophic flagellates in brown environments. Therefore, examining plastic responses of mixotrophic algae to browning is necessary to settle the bases for understanding the biogeochemistry, algal physiology and food web ecology of browning lakes.

In this study, we used *Cryptomonas* sp. (isolated from a clear water lake) as a model system for plastic responses to browning environments to ask the following questions: (i) is mixotroph growth negatively impacted by browning; (ii) does browning affect the use of light energy; (iii) does browning affect FA or sterol quality and quantity in mixotrophs; (iv) do mixotrophs change their photosynthetic pigment content with browning; and (v) which genes show differential expression between phototrophic, glucose-supplemented phototrophic and browning conditions? To answer these questions, we cultivated *Cryptomonas* sp. under three different control conditions (phototrophic, glucose-supplemented phototrophic and heterotrophic) in addition to five degrees of browning (DOC concentrations ranging from 1.5 to 90 mg C L^{-1}). During exponential growth, we performed a comparative transcriptome analysis followed by differential gene expression analysis between three browning treatments and two of our controls. The balance between phototrophic and heterotrophic activity was evaluated by the incorporation of ^{13}C-labelled carbon from $NaHCO_3$ into membrane lipids. At the end of the experiment, biomass analyses were carried out to observe differences in FAs, sterols and pigments between treatments.

2 | MATERIALS AND METHODS

2.1 | Strains, culture preparation and growing conditions

Cryptomonas sp. strain CPCC 336 was obtained from the Canadian Phycological Culture Centre, originally isolated from a clear-water lake, and thus was not readily adapted to brown waters. The algae

were maintained phototrophically in the authors' culture collection as stock cultures in MWC (Modified Wright's Cryptophyte) media (Guillard & Lorenzen, 1972) with some modifications (Stevčić et al., 2019; see Methods S1) at 18°C under light–dark cycle of 12:12 h (light intensity of 50–70 μmol quanta m^{-2} s^{-1}).

All experimental treatments described next were prepared in quadruplicate using our modified MWC media and grown using the same light conditions as the stock cultures. A photoautotrophic treatment (Control) was prepared using modified MWC media and *Cryptomonas* sp. inoculum. Two glucose-supplemented treatments (GLU and DARK) were prepared as Control and D-glucose (Sigma-Aldrich) was added to a final concentration of 5 mg C L^{-1}. One of these treatments was maintained in the dark during the course of the experiment (DARK treatment). Experimental DOC cultures (concentrations: 1.5, 10, 30, 50 and 90 mg C L^{-1}) were prepared using a DOC mix (500 mg C L^{-1}; see Methods S1), filtered lake water or a combination of both accounting for 18% of the culture's volume. The rest of the culture volume was MWC medium and the algae inoculum. All treatments were grown in 650-ml plastic culture flasks containing a final volume of 400 ml. To avoid carbon limitation, we weekly added 0.720 mg of carbon in the form of $NaHCO_3$ (to Control and DOC treatments) or glucose (to GLU and DARK) until the end of the experiment. All $NaHCO_3$ available in DOC treatments (initial MWC concentration and weekly additions) was enriched with 5% ^{13}C-$NaHCO_3$ (Sigma-Aldrich) for stable isotope analysis. Cell concentration was measured every 2–3 days using a flow cytometer (Guava easyCyte HT; Luminex).

2.2 | Bulk stable isotope analysis of phospholipid fraction

Cells were harvested on day 16 by filtration (50–100 ml of culture) through 3.0-μm cellulose nitrate membranes (Whatman, GE Healthcare), frozen at −80°C for 45 min, and freeze-dried overnight (three or four replicates). Total lipids were extracted with 3.75 ml chloroform/methanol/water (4:2:1) using sonication (10 min). After phase separation, solvents were evaporated at 50°C under a nitrogen stream, and 400 μl of chloroform was added to dried lipid samples. Phospholipid fractions of lipid extracts were obtained via solid-phase extraction using silica cartridges (500 mg, Bond Elut LRC—SI; Agilent). Cartridges were preconditioned with chloroform before the lipid extract was added. Chloroform and acetone (8 ml of each) were first used to elute nonpolar lipids, and the phospholipid fraction was eluted with 8 ml methanol. Chloroform fractions were stored at −20°C for sterol analysis. Phospholipids in methanol were dried at 50°C under a nitrogen stream, dissolved in methanol and added to a smooth-wall tin cup (D4057 Elemental Microanalysis). Tin cups were dried overnight under a fume hood, and the $\delta^{13}C$ values of these samples were analysed using a Thermo Finnigan DELTA^{plus}Advantage mass spectrometer (Thermo Electron) connected to a FlashEA 1112 Elementar Analyser. Birch leaves (*Betula pendula*) were used as internal standards during the run. In addition, three replicates of freeze-dried DOC mix (500 mg C L^{-1}) were analysed to get their signature $\delta^{13}C$ value.

2.3 | Fatty acid and sterol analysis

Algal cells were harvested (2–5 mg dried weight) on day 16 and lipids extracted as described above. After phase separation and evaporation at 50°C, 500 μl of acetone was added, and 40% of the sample was stored for carotenoid analysis (−20°C). The rest of the sample was evaporated and dissolved in 1 ml toluene, and FAs were transesterified overnight (50°C) using methanolic H_2SO_4 (1%, v/v). FA methyl esters were analysed with a gas chromatograph equipped with a mass detector (GC–MS; Shimadzu Ultra) using a DB-23 column (30 m × 0.25 mm × 0.25 μm; Agilent). Quantification of FAs was based on peak integration using GCSOLUTION software (version 2.41.00, Shimadzu; see Methods S1). Peak areas of FAs were corrected by using two internal standards (phospholipid FA C19:0 and free FA C23:0; Larodan) added before lipid extraction. Replicates of DOC mix (500 mg C L^{-1}) were extracted and analysed for FAs as controls. Only FAs that were not identified or present in trace amounts in the DOC mix were used for comparisons between treatments (referred to as algal FAs in the text). For percentage calculations and FA content, only the algal ω-3 (18:3ω-3, 18:4ω-3, 20:4ω-3, 20:5ω-3 and 22:6ω-3) and ω-6 (18:2ω-6, 22:5ω-6) FAs were used.

Stored nonpolar lipid extracts for sterol analysis were evaporated at 50°C under a nitrogen stream. For silylation, 100 μl of pyridine (Sigma-Aldrich) and 70 μl of N,O-bis[trimethylsilyltrifluoroacetamide] (BSTFA) with 1% (w) trimethylchlorosilane (TMCS; Sigma-Aldrich) were added to all samples and incubated at 70°C overnight. Trimethylsilyl (TMS) derivatives of sterols were analysed with a gas chromatograph (GC-2010 Plus, Shimadzu) coupled with a flame ionization detector (FID-2010 Plus, Shimadzu) using a Phenomenex Zebron ZB-5 HT Inferno column (30 m × 0.25 mm × 0.25 μm) for separation (see Methods S1). Identification of sterols was based on retention times using a plant sterol mix (Larodan). Quantification was done using a three-point calibration curve based on known standard concentrations. Concentration corrections of sterol samples were done using the internal standard 5-α-cholestane (Sigma-Aldrich).

2.4 | Chlorophyll-*a* and carotenoids

Samples for chlorophyll-*a* concentration analysis were taken on day 16. Samples (2 ml of culture) were filtered with GF/C filters (Whatman) and stored at −20°C for less than 1 month before chlorophyll was extracted using hot 94% ethanol (wt%). Throughout the process, samples were handled in dim light and sheltered from direct light exposure. For extraction, filters were immersed in 10 ml ethanol and incubated at 75°C for 5 min. The obtained solution was filtered through 0.45-μm pore size nylon syringe filters (Whatman) into a 1-cm quartz cuvette. Absorbance at wavebands of 665 and 750 nm was measured with a Shimadzu UV-1800 spectrophotometer.

Chlorophyll-*a* was quantified utilizing an absorption factor of 11.9 (SFS-ISO 10260:1992).

Stored carotenoid samples (previously obtained aliquots of total lipids) were separated and analysed via ultrahigh-performance liquid chromatography (Nexera, Shimadzu) coupled with a SPD-M20A diode array detector (Shimadzu) using a YMC carotenoid column (250×4.6 mm.D. S-5 μm; YMC). Identification of carotenoids was done using retention times and absorption spectra of observed peaks. Quantification of carotenoids was achieved by using a previously obtained factor between the peak areas of the sample internal standard Trans-β-Apo-8′-carotenal (Sigma Aldrich) and the target carotenoid (see Methods S1).

2.5 | RNA preparation and transcriptomic analysis

After 14 days of cultivation, 50 ml of every culture was centrifuged for 5 min (2500 *g*, 18°C), after which the supernatant was discarded. Cell pellets were resuspended in 700 μl of DNA/RNA Shield and homogenized in BashingBead (0.1 and 0.5 mm) lysis tubes (Zymo Research) using a TerraLyzer Cell Disruptor. RNA was extracted using a Chemagic 360 and the Chemagic Viral DNA/RNA 300 Kit H96 following the manufacturer's instructions (PerkinElmer). Extracted RNA was treated with DNAse I (Thermo Fisher Scientific), purified using Zymo spin-columns and quantified using a NanoDrop (Thermo Fisher Scientific). Samples were stored at −80°C.

Control, DOC 1.5, DOC 30, DOC 90, and GLU treatments were selected for transcriptomic analysis. RNA library generation, transcriptome sequencing, and annotation and differential expression analysis of the chosen treatments were conducted by Novogene. Briefly, three biological replicates of each treatment were sequenced with a NovaSeq 6000 (Illumina) to produce 150-bp paired-end reads. The targeted number of reads for each sample was 30 million. After removing reads with adaptor contamination or uncertain nucleotides, de novo transcriptome assembly (in the absence of a reference genome) was carried out by TRINITY 2.6.6 (Grabherr et al., 2011). Clustering of contigs based on shared reads was done with CORSET 4.6 (Davidson & Oshlack, 2014) to remove transcriptome redundance. The completeness of the transcriptome was assessed by the Benchmarking Universal Single-Copy Orthologs (BUSCO 3.0.2; Simao et al., 2015) with the eukaryota odb9 database. Differential expression analysis (*p*-value estimation) was carried out using DESEQ2 (Love et al., 2014). DESEQ2 was used to normalize readcount data and a negative binomial distribution was used for *p*-value estimation model. The BH procedure was used as a false discovery rate estimation method. We selected a threshold of 2-fold change in expression between treatment and our phototrophic control (while having a significant *p*-value) to be considered a differentially expressed transcript (DET: $|\log_2 \text{FoldChange}| > 1$ and $p < .05$).

For transcript functional annotation, we matched the DETs = s to the Kyoto Encyclopedia of Genes and Genome (KEGG) Orthology (KO) database using KAAS (KEGG Automatic Annotation Server) with an evalue threshold of $1e^{-5}$. Given that many annotated genes in the KO database had several matching DETs, we obtained the weighted average Log_2 fold-change expression change between treatments and Control for each KO gene ID. The weight of each transcript was assigned based on the transcript abundance value. To visualize changes in expression of genes involved in glycolysis, the tricarboxylic acid (TCA) cycle and photosynthesis, Figure 6 was prepared by manually selecting transcripts involved in these pathways. A Venn diagram of DETs was produced in R with the VENNDIAGRAM package (version 1.6.20).

2.6 | Statistics and stable isotope mixing model

Multiple comparisons were carried out with Dunnett's test comparing the results of Control against all other treatments. Before the analysis, homogeneity of variances was tested with Levene's test. Permutational multivariate analysis of variance (PERMANOVA) based on the Bray–Curtis distance matrix, as well as multivariate homogeneity of group dispersion (Anderson, 2006) were performed on FA, sterol and carotenoid contribution data (%) using treatment as the only factor. The limit of statistical significance in all tests was set to $\alpha = 0.05$. All statistical analyses were conducted using R (RStudio version 3.6.3) with either R base or VEGAN package (Oksanen et al., 2018).

A two-source carbon isotope mixing model, ISOERROR, version 1.04 (Phillips & Gregg, 2001), was used to assess how much of the phospholipid-derived fatty acids (PLFAs) from DOC treatments originated from labelled inorganic carbon (phototrophic) and how much from DOC (heterotrophic). DOC 1.5 was selected as the most phototrophic of all DOC treatments due to its negligible light limitation, and hence used as the $\delta^{13}C$ value of highest phototrophy. The mean proportion of phototrophy PLFA (fA = labelled inorganic carbon) by our mixotrophic algae was calculated using the following mixing model:

$$(\text{fA}) = (\delta\text{M} - \delta\text{B}) / (\delta\text{A} - \delta\text{B})$$

where δM represents the $\delta^{13}C$ values of DOC treatments PLFA, δA represents the $\delta^{13}C$ value of DOC 1.5, and δB represents the $\delta^{13}C$ value of DOC mix. Isotopic fractionation was not considered since the variation of $\delta^{13}C$ values of PLFA and DOC mix varied more than previously measured isotopic fractionation (e.g., −4‰; Bec et al., 2011) due to the ^{13}C-enrichment.

3 | RESULTS

3.1 | Browning and cell growth

To assess the effects of browning on the studied mixotrophic algae, five naturally occurring DOC concentrations ranging from low to very high (1.5, 10, 30, 50 and 90 mg C L^{-1}; Figure S1) were studied and compared to fully phototrophic cultures (Control). In addition,

we examined the effects of glucose supplementation under phototrophic and heterotrophic conditions (GLU and DARK treatments, respectively). DARK treatment showed limited initial growth, and cell numbers started to decline after 2 days of cultivation. Therefore, this treatment was ended 2 days earlier than in the other cultures, and no further analyses were carried out. Glucose supplementation under phototrophic conditions enhanced growth, leading GLU to ~35% higher cell densities than Control and the overall highest cell densities of all treatments (Figure 1a). DOC concentrations from 1.5 to 50 mg C L^{-1} did not have a large impact on the growth curve, and these cultures reached similar final cell densities. DOC 90 had the highest cell densities of all DOC treatments, surpassing Control and achieving comparable, but overall lower, cell densities to GLU. The analysis of per day increase in cell population number, measured as specific growth rate, revealed that GLU (0.14 ± 0.004 day^{-1}), DOC 90 (0.13 ± 0.003 day^{-1}) and DOC 10 (0.12 ± 0.004 day^{-1}) treatments grew faster than Control (0.11 ± 0.001 day^{-1}; Dunnett's test, $p < .05$), while no statistically significant differences were seen between the other cultures and Control (Figure 1b). In summary, our results with glucose supplementation show that our model algae cannot grow heterotrophically, but mixotrophic conditions enhance growth. In addition, since browning reduces the amount of available photosynthetically active light, our results from DOC treatments suggest a mechanism to bypass this reduction in light to sustain and even potentiate growth.

3.2 | Nutritionally important biomolecules

In view of the ecological importance of the nutritional quality of algae for higher trophic levels, we tested if browning leads to an alteration in the composition or concentration of FAs and sterols. Overall, 22 FAs and three sterols were identified and quantified. Because bacteria and the organic matter present in the DOC mixture used to prepare our DOC treatments contained FAs (see DOC extract preparation, Methods S1), we selected seven nutritionally important algal ω-3 and ω-6 FAs that were not present in the DOC mixture. The contribution of linolenic acid (LIN, 18:2ω-6), alpha-linolenic acid (ALA, 18:3ω-3), stearidonic acid (SDA, 18:4ω-3), 20:4ω-3, eicosapentaenoic acid (EPA, 20:5 ω-3), 22:5ω-6, and docosahexaenoic acid (DHA, 22:6 ω-3) to total algal FAs was affected by treatment. Of the total variance observed, 68% was explained by the factor treatment ($p < .05$, PERMANOVA; Figure 2a; Tables S1 and S2). Nevertheless, total ω-3 and ω-6 FA cell contents were not significantly different between Control (5.32 ± 0.95 and 0.40 ± 0.06 pg per cell ω-3 and ω-6, respectively) and the rest of the treatments (Figure 2b,c).

Sterol composition and content showed a strong response to browning. Brassicasterol and stigmasterolwere found in all treatments, but beta-sitosterol was only observable in the browning treatments (Figure 2d) and was highest for DOC 30 (7.4% of all sterols). Sterol concentration data showed that all DOC treatments had slightly higher total sterol concentrations than Control (0.44 ± 0.07 pg per cell). DOC 10 (0.92 ± 0.15 pg per cell), DOC 30 (0.97 ± 0.15 pg per cell) and DOC 90 (0.95 ± 0.38 pg per cell) had significantly higher total sterol concentrations (Figure 2e).

3.3 | Utilization of inorganic carbon

Since enhanced mixotrophy can increase the reliance of algae on organic carbon for growth, we tested if browning produces a change in the utilization of inorganic carbon. For this, we used isotopically

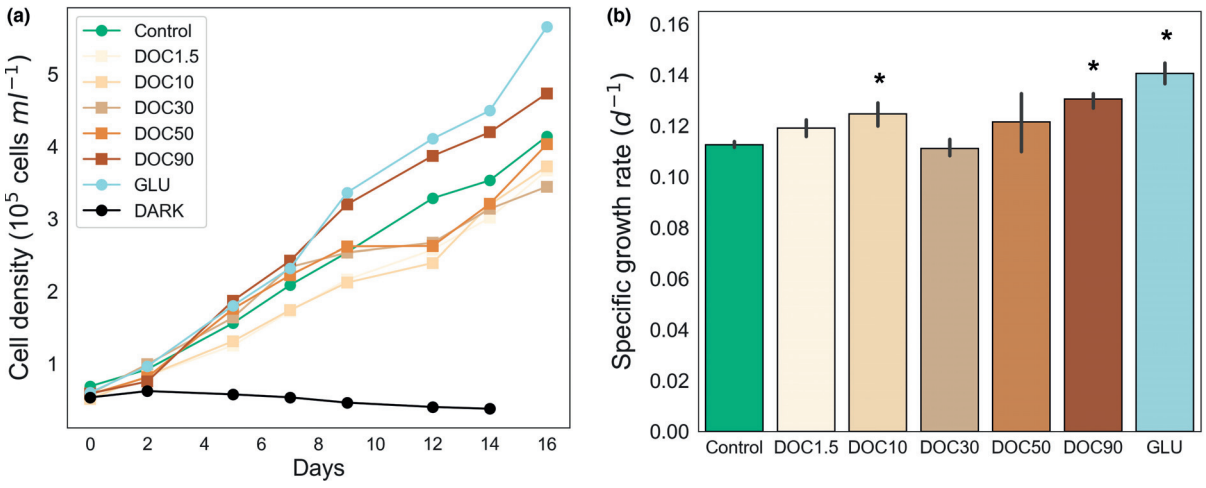

FIGURE 1 Cell growth under increasing DOC concentration and glucose supplementation. Cell density dynamics (a) and specific growth rate (b) of our mixotrophic algae model (*Cryptomonas* sp.) grown under phototrophic (control), glucose-supplemented phototrophic (GLU) or heterotrophic (DARK) conditions and under experimental concentrations of DOC. DOC concentrations ranged from 1.5 to 90 mg C L^{-1}. Values are presented as mean ± *SD* of three to four replicates. Statistically significant differences between one of the treatments and control are shown with an asterisk (Dunnett's test).

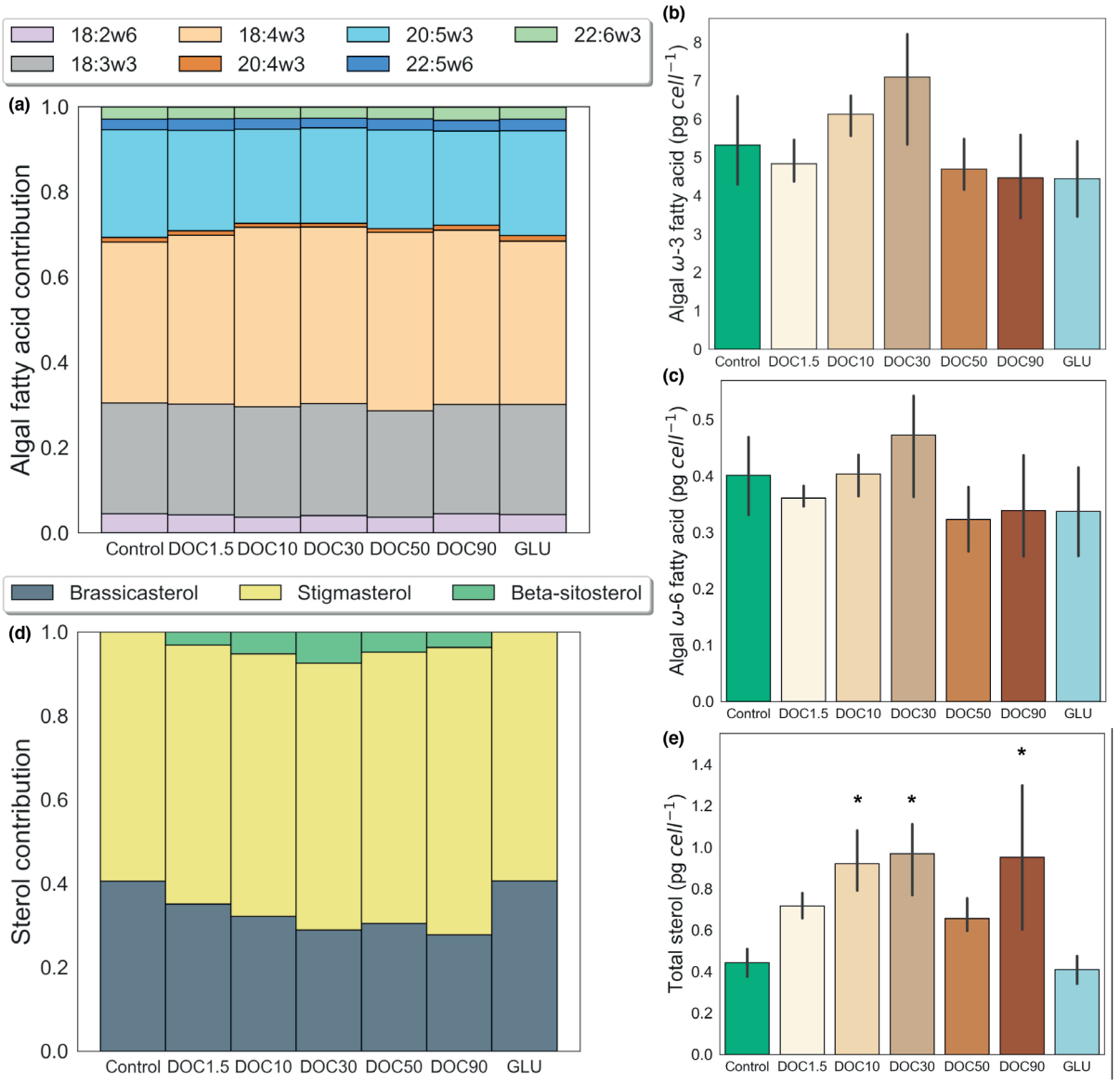

FIGURE 2 Changes in ecologically important biomolecules among DOC concentration increases or glucose supplementation (GLU). Algal fatty acid (a) and sterol (d) contribution to individual species of these groups of biomolecules. Total algal ω-3 and ω-6 fatty acid (b and c respectively) and sterol (e) content were normalized per cell. DOC concentrations ranged from 1.5 to 90 mg C L^{-1}. Values are presented as mean ± *SD* of three to four replicates for (a)–(c), while only the mean is given in contribution plots.

^{13}C-labelled sodium bicarbonate (^{13}C-$NaHCO_3$) in all browning treatments and followed its incorporation into the algal phospholipidic fraction. Phospholipids were used instead of total lipids, since phospholipids are rapidly degraded upon cell death and are therefore representative of viable biomass (Pinkart et al., 2002). Increases in the degree of browning led to a reduction in the incorporation of heavier carbon into phospholipids (Figure 3a). DOC 1.5 had the highest incorporation of ^{13}C (134.5 ± 7.1 δ^{13}C), whereas DOC 90 had the lowest (75.4 ± 8.1 δ^{13}C). Mixing model results using DOC 1.5 as the baseline for phototrophy (100% reliance on inorganic carbon) showed that increases in DOC were associated with increases in heterotrophy (Figure 3b). Accordingly, the lowest inorganic carbon incorporation was seen in DOC 90 (63 ± 2.9% of carbon use; Figure 3b). These results suggest that there is a rapid switch in the utilization of carbon from inorganic to organic sources with browning. Therefore, given the similar growth rates obtained for DOC treatments, mixotrophs may reduce their dependence on phototrophy to sustain growth.

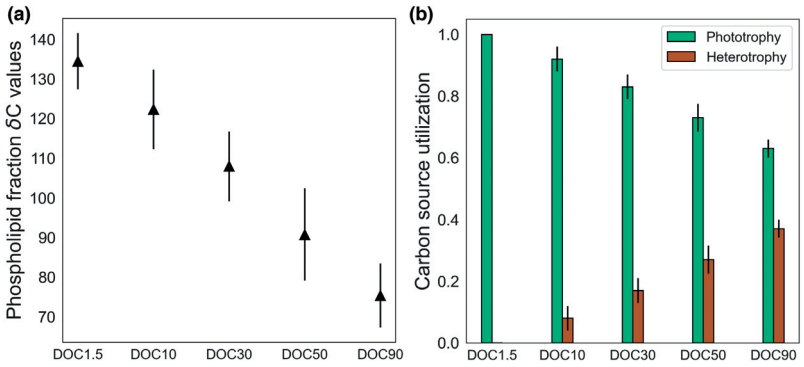

FIGURE 3 Utilization of inorganic (phototrophy) and organic (heterotrophy) carbon among changes in DOC concentration. Phospholipid fraction δC values (a) of 5% ^{13}C-NaHCO$_3$-enriched DOC treatments among increasing DOC concentrations. Mixing model results of carbon source utilization (b) using DOC 1.5 as the most phototrophic of all DOC treatments (see Section 2). Values are presented as mean ± *SD* of three to four replicates.

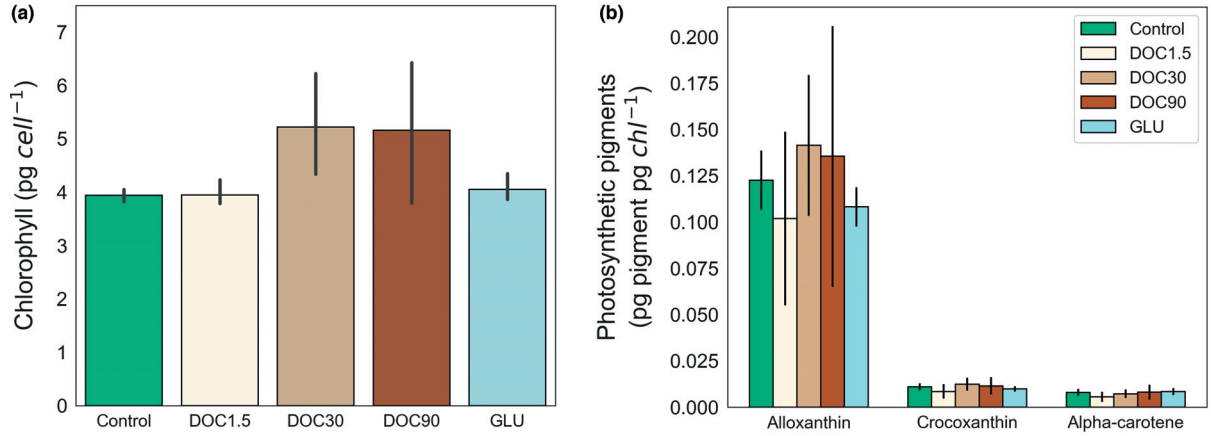

FIGURE 4 Pigment variation in response to increases in DOC concentration and glucose supplementation (GLU). Total chlorophyll-*a* normalized per cell (a) and carotenoid content relative to chlorophyll-*a* (b) of three DOC treatments and GLU compared to phototrophic conditions (control). Values are presented as mean (± *SD* in a and c) of three to four replicates. Statistically significant differences between one of the treatments and control are shown with an asterisk (Dunnett's test).

3.4 | Chlorophyll and carotenoids

Since manipulation of photosynthetic pigments can increase light energy harvesting under reduced light availability, we then investigated the chlorophyll-*a* and carotenoid content per cell of Control, GLU and three of the DOC treatments (1.5, 30 and 90 mg C L^{-1}). Chlorophyll-*a* concentration per cell was not affected by browning or glucose supplementation compared to Control (3.94 ± 0.09 pg per cell), despite slightly higher average contents in DOC 30 and DOC 90 (Figure 4a). Alloxanthin, crocoxanthin and alpha-carotene were the three identified carotenoids in all samples (Figure 4b). Alloxanthin was the highest contributing carotenoid (>80%) of all quantified carotenoids, followed by crocoxanthin (~8%) and alpha-carotene (~5%). The contribution of individual carotenoids did not vary between treatments ($p > .05$, PERMANOVA; Table S1). Carotenoid content relative to chlorophyll-*a* was not affected by browning or glucose supplementation (Figure 4b). As expected, alloxanthin relative to chlorophyll-*a* content was the highest of all carotenoids (0.151 ± 0.05 pg pg^{-1} in Control), highlighting this carotenoid as the main light-harvesting accessory pigment.

3.5 | Transcriptomic analysis

To gain insight into genome-wide gene expression changes that modulate the adaptation of mixotrophs to browning, we conducted a de novo RNA sequencing (RNA-seq) analysis of five of our treatments (Control, DOC 1.5, DO 10, DOC 90 and GLU). More than 30 million raw reads were obtained per sample and the final transcript length varied from 301 to 40,155 nucleotides with a mean length of 1266 nucleotides (Tables S3 and S4). Transcriptome assembly identified ~250,000 unique transcripts (Table S4). The ompleteness of the assembled transcriptome was low in BUSCO (79.4% missing; Table S5). Nevertheless, these results were not surprising given the evolutionary divergence of cryptophytes and the fact that many of the genes in the Eukaryotic database are absent in many highly contiguous algal genomes (Curtis et al., 2012; Hanschen et al., 2020). No genomic data are currently available for *Cryptomonas* sp., but the related cryptophyte *Guillardia theta* showed the highest species similarity (49.3%) based on transcriptome species classification (Figure S2). Functional annotation of the assembled transcriptome exhibited a large number of novel transcripts that did not match with

any sequence in available databases (51.1% of the transcriptome). To examine the effects of browning and glucose supplementation on gene expression, we identified all DETs between these treatments and Control. Transcripts were only considered differentially expressed if the change in transcript abundance between treatment and Control was 2-fold or more in either direction while having a significant *p*-value (DET: $|\log_2 \text{FoldChange}| > 1$ and $p < .05$).

Close to a fifth of all transcripts in the transcriptome were differentially expressed upon browning or glucose supplementation (51,427 out of 254,822 total transcripts; Figure 5). Increases in the degree of browning had a strong effect on gene expression, with an almost 2-fold increase in the number of DETs between DOC 1.5 and DOC 30 (8273 and 17,585 transcripts, respectively) and DOC 30 and DOC 90 (17,585 and 30,187 transcripts, respectively; Figure 5a). In comparison, glucose supplementation led to a change in the expression of 10,358 transcripts. All treatments shared >1200 DETs while >500 transcripts were differentially expressed in DOC treatments alone (Figure 5b). Overall, more than 55% of each treatment's DETs were up-regulated, and DOC 90 presented the highest percentage of up-regulated transcripts (>75% of all DETs).

For further analysis, we carried out a functional enrichment analysis using the KO database. Since we did not have a genomic sequence to map the assembled transcriptome against, many annotated genes in the KO database presented several matching transcripts. Therefore, to obtain the expression levels of individual genes, we used the weighted average $\log_2$ fold-change expression with respect to Control of all identified transcripts matching one KO identifier. Given our previous results showing changes in inorganic carbon utilization along the DOC gradient, we focused on transcriptomic evidence of changes in metabolic pathways related to phagocytosis, light energy conversion and respiration. KO classifications (gene groups) specific to phagotrophic activity (phagosome, lysosome and endocytosis) had a higher number of up-regulated compared to down-regulated genes in all DOC treatments (Figure 5c).

Moreover, the number of up-regulated differentially expressed genes (DEGs) involved in phagotrophic activity increased along the browning gradient from 41 to 72 with an average $\log_2$ fold-change increase of 4.49 9.64 in DOC 1.5 to DOC 90, respectively. In contrast, GLU had a higher number of down- than up-regulated DEGs in the same KO classifications (eight up- and 14 down-regulated genes) and an average $\log_2$ fold-change of −0.37. These results suggest that browning, but not glucose supplementation, stimulates phagotrophic activity.

We then mapped RNA abundance changes upon DOC increases and glucose supplementation to metabolic genes involved in glycolysis, the TCA cycle and photosynthesis (Figure 6). Photosynthetic

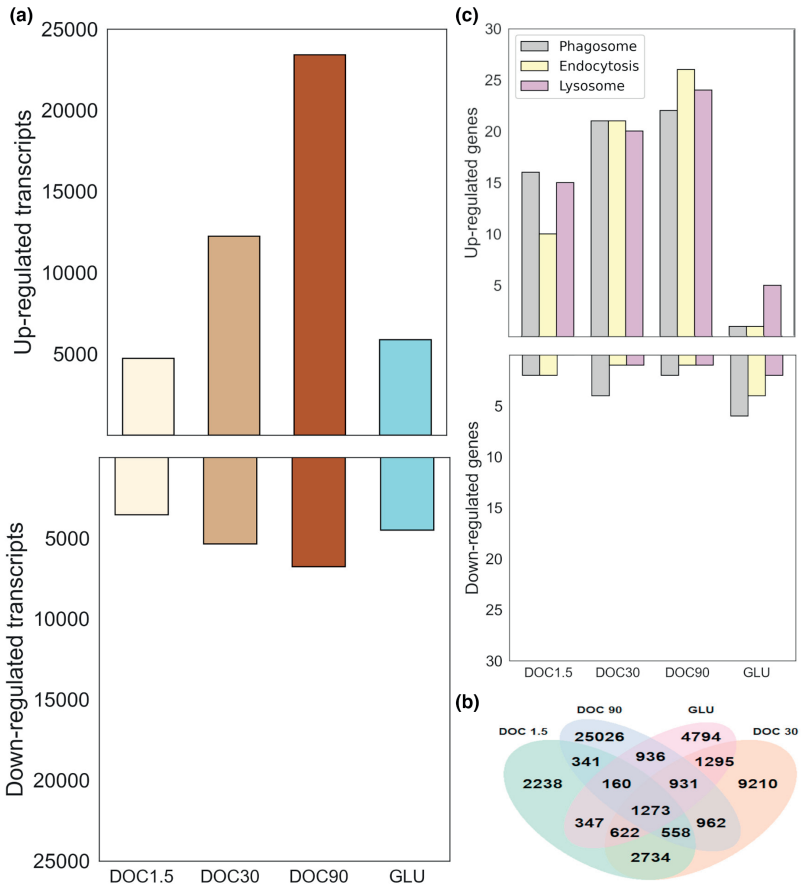

FIGURE 5 Transcriptome responses to increases in DOC concentrations and glucose supplementation (GLU). Differentially expressed transcripts (a) were considered all transcripts that were more than 2-fold up- or down-regulated relative to the phototrophic control (control) samples having a $p < .05$ (see Section 2). Venn diagram (b) showing the overlap of differentially expressed transcripts (up- and down-regulated) between DOC and GLU treatments. DEGs in phagotrophy-related gene categories (phagosome, endocytosis and lysosome; c) represent phagotrophic activity among increases in DOC or glucose supplementation. Functional annotation of transcripts into phagotrophy-related gene categories was done using the KO database (Data Set S1). To obtain the number of unique genes in each category, weighted averages of all differentially expressed transcripts between each treatment and control matching the same gene ID in the KO database were used. Data represent the means of three biological replicates.

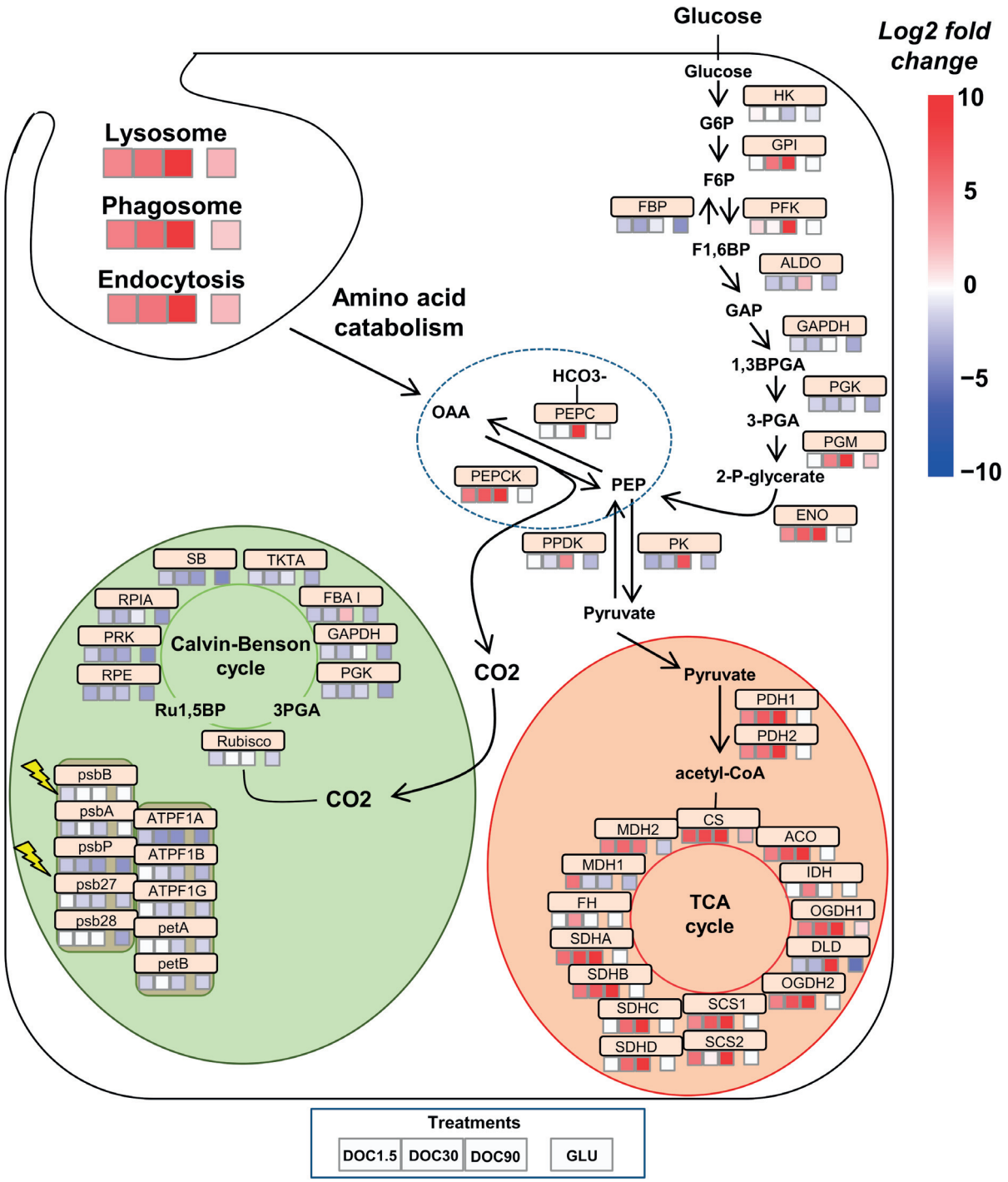

FIGURE 6 Adaptations in photosynthesis, glycolysis and TAC cycle among increases in DOC concentration and glucose supplementation (GLU). Functional annotation of genes was done with the KO database. Squares beneath gene names represent Log_2 fold-change values of the respective treatment and the phototrophic control. Log_2 fold-change values were obtained with the weighted average of all transcripts matching the same KO gene ID. Under gene groups, lysosome, phagosome and endocytosis, squares represent the average Log_2 fold-change value of all up-regulated genes in that category. Plastid is indicated in green, mitochondrion in red and cytosol in white. Data represent the means of three biological replicates. Fold-change values and gene IDs are in Data Set S2.

transcripts (enzymes of the Calvin–Benson cycle and electron transport chain complexes) were down-regulated in DOC and GLU treatments compared to Control. Interestingly, only photosystem II (PSII) components, proton-translocating ATPase, cytochrome b6f and ferredoxin were down-regulated, while no gene matching to photosystem I was differentially expressed between treatments. TCA cycle enzyme transcripts were highly up-regulated in all DOC treatments, while glucose supplementation did not affect their expression compared to the Control. Transcript abundance of glycolytic enzymes presented an overall increase along the browning gradient, although no general pattern of up- or down-regulation of the complete pathway was seen.

Similarly to TCA cycle genes, glucose supplementation did not produce significant expression changes in glycolytic enzymes. The two phosphoenolpyruvate (PEP) enzymes suggested to play a critical role in bacterial ingestion by mixotrophs (González-Olalla et al., 2021; Liu et al., 2016), carboxykinase (PEPCK) and carboxylase (PEPC), had contrasting expression patterns among treatments. PEPCK was up-regulated in all DOC treatments compared to Control, and its transcript abundance was increased along the browning gradient. In contrast, PEPC was only up-regulated in DOC 90, and no change in expression was found in the other two DOC treatments. Expression of none of these enzymes was affected by glucose supplementation compared to Control. In summary, our transcriptomic results indicate apparent differences in carbon metabolism upon browning and glucose supplementation and support our previous results on the decreasing reliance on phototrophy with browning.

4 | DISCUSSION

The impact of browning processes in aquatic ecosystems includes a shift from autotrophic to heterotrophic-based basal production (Creed et al., 2018). This study provides physiological, biochemical and transcriptomic evidence supporting such a prediction, indicating that browning leads to an increased reliance on heterotrophically acquired energy in our model mixotroph (*Cryptomonas* sp.). Our growth rate results validated using the selected algae species as a model mixotroph since glucose supplementation in the presence of light enhanced growth, as previously seen with other mixotrophic species (Smith et al., 2015; Zhang et al., 2021). In addition, fully heterotrophic conditions could not sustain growth, highlighting the need for basal levels of phototrophy for survival. Increases in water colour, and its associated reduction in light availability (Graneli, 2012), did not impair growth compared to fully phototrophic conditions. Conversely, two of our browning treatments (DOC 10 and DOC 90) had higher specific growth rates than under purely phototrophic conditions. Nevertheless, since increases in growth rate were modest with browning, we do not believe that such differences alone explain the dominance of mixotrophic flagellates observed in highly brown environments (Bergström et al., 2003; Deininger et al., 2017).

Given that increases in water colour did not negatively impact growth, we investigated if inorganic carbon fixation is altered by browning in the studied mixotrophic algae. Our stable isotope analysis of phospholipid fractions showed that increases in water colour come with a reduction in inorganic carbon utilization for growth. Furthermore, as demonstrated by our transcriptomic results, the down-regulation of genes related to photosynthesis further supports this claim and indicates a decreased reliance on phototrophy with browning. Notably, even our lowest DOC concentration (DOC 1.5) showed a down-regulation of photosynthetic genes. These results suggest that when organic carbon is available, mixotrophs may rapidly capitalize on their ability to increase reliance on heterotrophy to sustain growth, possibly to decrease the high energetic costs of maintaining photosynthetic machinery (Raven, 1995). Such changes in the balance between phototrophy and heterotrophy with browning could potentially impact oxygen production and carbon cycling in mixotrophic-dominated environments. Nevertheless, further studies are needed to observe how oxygen production is affected by increases in water colour.

Surprisingly, despite a reduction of phototrophy with browning, we did not observe a decrease in the chlorophyll or carotenoid content per cell. Previous studies on plastic responses of chlorophyll levels to mixotrophy suggest that, together with the down-regulation of photosynthesis, there is a reduction in the production or enhanced degradation of chlorophyll (González-Olalla et al., 2021; Roth et al., 2019; Wilken et al., 2014). Nevertheless, these studies were carried out using algae species that can grow fully heterotrophically; hence, our model mixotroph (which requires light to survive) might have specific needs to maintain basal photosynthetic function.

Enhanced expression of genes associated with phagotrophy (lysosome, endosome and phagosome KO categories) with browning, but not with glucose supplementation, suggests that heterotrophy was supported by predation of bacteria. These results agree with mesocosm experiments by Wilken et al. (2018), where mixotrophs showed higher bacterial predation along with temperature and water colour increases. Although the phagotrophy-related genes used in our analysis have been identified in macrophages and could have different functions in algae (Bohdanowicz & Grinstein, 2013; Flannagan et al., 2012; Settembre et al., 2013), other laboratory studies have observed expression of these groups of genes in algae growing under mixotrophic conditions (Li et al., 2021; Lie et al., 2017; McKie-Krisberg et al., 2018). In addition, the high expressions observed for key phagotrophy enzymes (PEPCK and PEPC) in our browning treatments agree with previous studies on phagotrophic activity of algae (González-Olalla et al., 2021; Liu et al., 2016; McKie-Krisberg et al., 2018). PEPCK is believed to be the enzyme by which degraded bacterial proteins are channelled to energy production (González-Olalla et al., 2021; Liu et al., 2016). Following this, oxidation of organic substrates has been shown to accelerate in the presence of bacterial prey (González-Olalla et al., 2021), which matches the up-regulation of the TCA cycle genes observed in our experiment with browning.

The availability and quality of FAs and sterols in phytoplankton have been repeatedly demonstrated as necessary for the growth and reproduction of zooplankton (Brett & Müller-Navarra, 1997;

Martin-Creuzburg et al., 2009; Peltomaa et al., 2017). This has great ecological importance since zooplankton have a key position in aquatic food webs, linking the flow of dietary energy and essential biomolecules from phytoplankton to fish (Taipale et al., 2016). The FA concentration remained unaffected in our experiment while the sterol content and composition varied between browning and fully phototrophic conditions. Since the switch from phototrophy to heterotrophy has been previously characterized by plastic changes in both FAs and sterols in algae (Boëchat et al., 2007), it is possible that particular FAs and sterols are required for structural adaptations that occur during these transitions. We hypothesize that the higher phagotrophic activity associated with browning could potentially explain the differences in composition and content observed in sterols. We can only speculate that sterol requirements for our glucose-supplemented treatment were not different from fully phototrophic conditions, explaining why there were no differences between these treatments. Our results suggest that browning in mixotroph-dominated environments should not reduce the availability of ecologically important biomolecules at a cellular level. Nevertheless, further studies with other mixotrophic species are needed to observe if the regulatory strategies seen in *Cryptomonas* sp. with browning are common or diversified among mixotrophs.

Overall, we report the metabolic plastic changes, and the possible ecological implications, behind the success of a model mixotroph in browning environments. As observed in our study, metabolic plasticity allows *Cryptomonas* sp. to thrive in low light environments, where obligated phototrophs are limited by photosynthetic output. Furthermore, we showed that an enhanced reliance on bacterial prey accompanies the transition to mixotrophic growth; hence, the consequences of such a shift in aquatic ecosystems could signify higher CO_2 exportations but increased reliance of whole food webs on bacterial production. Further studies could look more directly into the biogeochemical consequences of phototrophy to mixotrophy transitions. In addition, since we studied the plastic adaptations of a mixotroph isolated from a clear-water lake, investigating the differences between evolved and plastic effects of browning is important in understanding the genetic architecture necessary for algae to thrive in browning environments.

AUTHOR CONTRIBUTION

M.L.C., P.S., E.P. and S.J.T. planned and designed the research. M.L.C., P.S. and C.R. performed the analysis. M.L.C. analysed the data. M.L.C., P.S., E.P. and S.J.T. interpreted the data. M.L.C. wrote the manuscript in collaboration with P.S., E.P., C.R. and S.J.T.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Raw transcriptomic sequence reads, as well as processed transcriptomic data, are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession no. "E-MTAB-11370." Raw phenotypic data and the mixing model used are available in the Data Set S3. All data as exportable files and data analysis files necessary to replicate the results presented in this study are available at https://doi.org/10.17011/jyx/dataset/80823. A comprehensive description of the content of each file, and the description of each Supporting Information data set can be found in the same DOI under the name "Metadata file."

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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II

TEMPERATURE, PHOSPHORUS, AND SPECIES COMPOSITION WILL ALL INFLUENCE PHYTOPLANKTON PRODUCTION AND CONTENT OF POLYUNSATURATED FATTY ACIDS

by

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ORIGINAL ARTICLE

Temperature, phosphorus and species composition will all influence phytoplankton production and content of polyunsaturated fatty acids

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Temperature increases driven by climate change are expected to decrease the availability of polyunsaturated fatty acids in lakes worldwide. Nevertheless, a comprehensive understanding of the joint effects of lake trophic status, nutrient dynamics and warming on the availability of these biomolecules is lacking. Here, we conducted a laboratory experiment to study how warming (18–23°C) interacts with phosphorus (0.65–2.58 μM) to affect phytoplankton growth and their production of polyunsaturated fatty acids. We included 10 species belonging to the groups diatoms, golden algae, cyanobacteria, green algae, cryptophytes and dinoflagellates. Our results show that both temperature and phosphorus will boost phytoplankton growth, especially stimulating certain cyanobacteria species (*Microcystis* sp.). Temperature and phosphorus had opposing effects on polyunsaturated fatty acid proportion, but responses are largely dependent on species. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) synthesizing species did not clearly support the idea that warming decreases the production or content of these essential polyunsaturated fatty acids. Our results suggest that warming may have different effects on the polyunsaturated fatty acid availability in lakes with different nutrient levels, and that different species within the same phytoplankton group can have contrasting responses to warming. Therefore, we conclude that future production of EPA and DHA is mainly determined by species composition.

KEYWORDS: phytoplankton; polyunsaturated fatty acids; lake; climate change; temperature; phosphorus

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INTRODUCTION

Lakes respond quickly to environmental change by altering their physical, chemical and biological properties, making estimations about the fate of these ecosystems complex (Adrian *et al.*, 2009). Temperatures across the globe are expected to increase due to climate change (IPCC, 2021) leading, in theory, to more productive aquatic ecosystems (Falkowski and Raven, 2007). Nevertheless, lake nutrient status and dynamics, morphological characteristics and light availability are likely to play a role in modulating the effects of temperature increases in these ecosystems (Adrian *et al.*, 2009; Jennings *et al.*, 2009; Björnerås *et al.*, 2017; Tabari, 2020). In high-latitude regions of the northern hemisphere, lakes are observed in high frequencies and provide significant ecosystem services in addition to habitats for wildlife (Chapin *et al.*, 2004). In northern areas, climate change is expected to increase precipitations, facilitating the run-off of nutrients (phosphorus and nitrogen) and dissolved organic carbon from catchment areas. This can result in eutrophication and browning of surface waters (de Wit *et al.*, 2016, Ruosteenoja *et al.*, 2016). Therefore, understanding how temperature increases and nutrients interact to shape lake responses is key when making estimations about the fate of northern lakes.

Phytoplankton provides aquatic food webs with energy, high-quality biochemical compounds and minerals (Sterner and Hessen, 1994; Peltomaa *et al.*, 2017). Temperature and nutrient increases have been shown to alter phytoplankton total biomass, community structure and the biochemical composition of individual cells (Adrian *et al.*, 2006; Rosenzweig *et al.*, 2007; Winder and Hunter, 2008; Taipale *et al.*, 2019). This is especially important when considering the availability of certain micronutrients present in phytoplankton that are essential for higher trophic levels. For example, long-chain polyunsaturated fatty acids (LC-PUFAs) such as eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3) are only produced by certain phytoplankton taxa but are required for the appropriate development and reproduction of consumers (Arts *et al.*, 2001; Parrish, 2009). Consequently, phytoplankton community alterations and changes in cellular contents reducing LC-PUFA availability can have cascading effects for higher trophic levels (Ahlgren *et al.*, 1992; Lang *et al.*, 2011; Taipale *et al.*, 2013). Currently, it is hypothesized that water warming will decrease PUFA availability, in particular EPA and DHA (Hixson and Arts, 2016; Colombo *et al.*, 2019), due to the overall decrease in fatty acid unsaturation degree observed across temperature gradients (Sepúlveda and Cantarero, 2022). These observations are conceptually validated by the homeoviscous adaptation theory (Sinensky, 1974), which states that at high temperatures saturated fatty acids give stability to cellular membranes. Over large temperature gradients, we believe that this theory holds since membranes originally adapted to cold environments are unstable at high temperatures. Nevertheless, nutrients such as phosphorus and nitrogen can also strongly modulate PUFAs (Su *et al.*, 2016; Ghafari *et al.*, 2016; Wang *et al.*, 2019), EPA and DHA (Xu *et al.*, 2001; Khozin-Goldberg and Cohen, 2006; Ren *et al.*, 2012; Matsui *et al.*, 2020) due to their participation in cellular metabolism and synthesis of certain lipid classes (Van Mooy *et al.*, 2009). Therefore, within the temperature increase expected with climate change, nutrients could play a significant role in the availability of PUFAs, leading to divergent scenarios than previously proposed (Hixson and Arts, 2016; Colombo *et al.*, 2019).

In lakes, phosphorus is a key macronutrient strongly associated with phytoplankton growth (Schindler, 1977). Given that phosphorus is a building block of membrane lipids (Van Mooy *et al.*, 2009; Cañavate *et al.*, 2016), and cell growth is interconnected with lipid metabolism (Tsai *et al.*, 2014), phosphorus concentration modulates phytoplankton PUFA, EPA and DHA availability (Khozin-Goldberg and Cohen, 2006; Ren *et al.*, 2012; Matsui *et al.*, 2020). No general effect of increasing phosphorus on phytoplankton LC-PUFAs has been observed, pointing to the diversity of phytoplankton life histories (Lubchenco and Cubit, 1980; Cock *et al.*, 2014) and highlighting that different species can present contrasting responses to changes in this nutrient (Adrian *et al.*, 2006). To date, most studies centred in the effect of phosphorus on phytoplankton LC-PUFAs have focused on nutrient depletion due to its applications in biotechnological processes (Khozin-Goldberg and Cohen, 2006; Ghafari *et al.*, 2016; Matsui *et al.*, 2020; Rawat *et al.*, 2021). Unfortunately, such an experimental approach completely overlooks phytoplankton responses to variations in phosphorus under non-depleted conditions, which could uncover valuable information about how differences in trophic status could affect LC-PUFAs availability in lakes.

We tested how simultaneous increases in temperature and phosphorus affect the growth, PUFAs and the LC-PUFAs (EPA and DHA) of 10 phytoplankton species common to northern lakes from six different phytoplankton groups. For this purpose, we use low (18°C) and high (23°C) growing temperatures combined with low (LP) and high (HP) available phosphorus to measure how phytoplankton growth rate, PUFA proportion, EPA and DHA content and production (measured as daily gain) are affected by these physicochemical changes. The experimental design was fully factorial. The objective

of this study was to investigate the interaction between temperature increase and phosphorus in PUFA and LC-PUFA availability. Our phosphorus treatments served as a proxy to study the effect of increasing temperature at different trophic status, as well as the effects of increasing phosphorus at different temperatures. We hypothesize that the effect of increasing temperature on phytoplankton LC-PUFA is dependent on phosphorus concentration (Khozin-Goldberg and Cohen, 2006; Ren *et al.*, 2012; Matsui *et al.*, 2020) and that there are large differences in responses between phytoplankton species due to their different life histories (Lubchenco and Cubit, 1980; Adrian *et al.*, 2006; Cock *et al.*, 2014).

MATERIALS AND METHODS

Strains, culture preparation and growing conditions

Ten species from the phytoplankton groups diatom (*Cyclotella* sp. and *Melosira* sp.), chrysophyte (*Synura* sp. and *Uroglena* sp.), cyanobacteria (*Microcystis* sp. and *Synechococcus* sp.), green algae (*Chlamydomonas reinhardtii* and *Desmodesmus maximus*), cryptophyte (*Rhodomonas* sp.) and dinoflagellate (*Peridinium cinctum*) were acclimatized to low phosphorus and 18°C before the start of the experiment. For such purposes, phytoplankton species were maintained autotrophically in the authors' culture collection as stock cultures in phosphorus-limited MWC (Modified Wright's Cryptophyte) media (Guillard and Lorenzen, 1972), at a phosphorus concentration of 6.46 μM, at 18°C under a 12:12-h light–dark cycle (light intensity of 100–125 μmol quanta m^{-2} s^{-1}). The experiment was divided into two halves to ensure proper experimental handling given the large number of phytoplankton cultures (120 cultures in total). In the first half cyanobacteria, green algae and cryptophytes were grown in 250 mL plastic culture flasks containing a final volume of 175 mL composed of 75 mL of phytoplankton stock and 100 mL of MWC. Experimental phosphorus-modified WC was prepared according to the treatment as low phosphorus (LP: 0.65 μM) and high P (HP: 2.58 μM). Phosphorus was added weekly to maintain cultures at their respective concentrations (assuming that phosphorus was zero at the moment of addition) to simulate consistent concentrations and avoid the effect of phosphorus depletion. Phytoplankton were grown in FH-130 (Taiwan Hipoint) growth chambers set a 18° and 23°C with a 12:12-h light–dark cycle and a light intensity 91–132 μmol quanta m^{-2} s^{-1}. In the second half of the experiment, diatoms, golden algae and dinoflagellates were grown in 600 mL plastic culture flasks containing a final volume of 400 mL composed of 100 mL of phytoplankton stock and 300 mL of the same experimental MWC as before. Growth chamber conditions, as well as phosphorus additions, were the same as for the first half of the experiment. In this half of the experiment, larger flasks were used to ensure enough biomass due to the lower biomass obtained from the stock cultures. The experimental design was fully factorial and each phytoplankton species in each treatment was prepared in triplicates in both halves of the experiment. Cell concentration was measured every 2–3 days using a flow cytometer (Guava easyCyte HT; Luminex). Experiment was terminated individually for each species once they reached stationary growth phase. Growth rate (day^{-1}) was calculated from the change in cell density during the exponential growth phase according to the formula: growth rate = $\ln(N_2/N_1)/(t_2 - t_1)$, where N_2 is the maximum measured cell density, N_1 is the cell density at Day 0 of the experiment and $(t_2 - t_1)$ is the time between the start of the experiment and the day where N_2 was measured.

Fatty acid analysis

Once all cultures of the same phytoplankton species reached stationary phase, phytoplankton cells were harvested by filtration through 3.0 μm cellulose nitrate membranes (Whatman, GE Healthcare), obtaining between 0.3 and 7.5 mg dry weight, depending on the species. Total lipids were extracted, and FA identified and analyzed as previously described (Calderini *et al.*, 2022) without dividing the sample into different fractions. Shortly, total lipids were extracted with chloroform/methanol/water (4:2:1) using sonication (10 min). After evaporation of solvents under a nitrogen stream, 1 mL toluene was added, and fatty acids were transesterified overnight (50°C) using methanolic H_2SO_4 (1%, v/v). FA methyl esters were analyzed with a gas chromatograph equipped with a mass detector (GC–MS; Shimadzu Ultra) using a DB-23 column (30 m × 0.25 mm × 0.25 μm; Agilent). Quantification of FAs was based on peak integration using gcsolution software (version 2.41.00, Shimadzu). Peak areas of FAs were corrected by using two internal standards (phospholipid FA C19:0 and free FA C23:0; Larodan) added before lipid extraction.

Data analysis

The value of PUFA proportion was obtained by dividing the content of all fatty acids containing two or more unsaturations by the sum of mono- and saturated fatty acids. Univariate analysis of variance (dependent variable: growth rate, PUFA proportion, EPA and DHA content and daily gain) was done with ANOVA, and

equality of variance was checked with Bartlett's test. Pairwise comparisons were carried out with Tukey's honestly significant difference test. If data presented a significant Bartlett's test (unequal variances), Kruskal–Wallis rank sum test was performed to assess the effects of the studied treatments. Non-parametric pairwise comparisons were also carried out with Kruskal–Wallis test using Bonferroni correction. Permutational multivariate analysis of variance (PERMANOVA) based on the Bray–Curtis distance matrix and multivariate homogeneity of group dispersion (Anderson, 2006) were performed on multivariate fatty acid composition (proportion of each FA) data using treatment, group or species as factors. Due to the collapse of *Synura* sp. LP cultures (18° and 23°C) before the end of the experiment, these treatments were taken out of the analysis and only *Synura* sp. HP (18° and 23°C) cultures were analyzed with ANOVA. ANOVA and PERMANOVA analyses were carried out to determine the overall effect of temperature and phosphorus on all studied species, excluding the data obtained for *Synura* sp cultivated in LP treatments. Non-metric multidimensional scaling (nMDS) was employed to visualize multivariate FA patterns in response to changes in temperature and phosphorus across the studied phytoplankton species. The limit of statistical significance in all tests was set to $\alpha = 0.05$. All statistical analyses were conducted using r (RStudio version 4.0.5) with either R base or vegan package (Oksanen *et al.*, 2018). Given the limitation of *P*-values as indicators of effect size (Wasserstein *et al.*, 2019), we used Glass' Δ (Glass *et al.*, 1981) as a fair estimate of effect size of treatments (Lin and Aloe, 2020). This estimate standardizes the difference in mean values between a control and a test group with the standard deviation observed in the control. We present such values in this study as heatmaps, where for each comparison between two treatments, the first denoted treatment is considered as control to calculate Glass' Δ.

RESULTS

Effect of temperature and phosphorus on growth rate

Across the studied phytoplankton species, growth rates varied close to one order of magnitude between the slowest (*Rhodomonas*: ∼0.06 day^{-1}) and the fastest (*Uroglena*: ∼0.30 day^{-1}) growing (Fig. 1; Fig. S1). *Synura* showed limited initial growth in LP, and after 2 days of cultivation, cell numbers started to decline (Fig. S1A). Therefore, only *Synura*'s HP treatments (18° and 23°C) were included in the rest of the analysis presented in this study. Phosphorus had a significant effect on growth rate across all species (ANOVA, Table S1), whereas no significant effect was observed for temperature. When including phytoplankton group or species factors in the model, phosphorus affected the growth rate significantly, although it explained only ∼6% of the variance (ANOVA; Table S1). At the species level, *Melosira* and *Uroglena* were not significantly affected by changes in phosphorus and temperature (Table S1), although positive effect sizes of temperature and phosphorus increases were seen in *Melosira*. Among the significantly affected species, phosphorus explained, on average ∼42% of the observed variance with the green algae *Chlamydomonas* presenting the highest explained variance (>90%). Temperature explained slightly less variance (∼39%) in growth rates than phosphorus, and the other studied green algae, *Desmodesmus* had the highest (95%) explained variance. Overall, increases in temperature and phosphorus had a positive effect on growth rate with highly variable effect sizes between species (average Glass $\Delta > 0$ for all treatments comparisons; Fig. 2B).

Effect of temperature and phosphorus on fatty acids

In total 40 fatty acids were identified and quantified across all 10 phytoplankton species. As expected, the high nutritional value LC-PUFAs EPA and DHA were present in 5 and 6 of the studied phytoplankton species (respectively) corresponding to the species *Melosira*, *Cyclotella* (diatoms), *P. cinctum* (dinoflagellate), *Uroglena*, *Synura* (golden algae) and *Rhodomonas* (cryptophyte) (Table S2). The effect of phosphorus on fatty acid profiles was modest and only significant at the species level, whereas no effect of temperature was observed (Fig. 2; PERMANOVA; Table S3). Since temperature is considered one of the main controllers of fatty acid unsaturation degree, we investigated how the proportion of PUFAs was affected with our treatments. Again, no significant effect of temperature or phosphorus was seen across all phytoplankton or phytoplankton groups (Fig. 3A and B; Table S4). When including the species term in our analysis, phosphorus had a significant effect on PUFA proportion (Table S4). At the species level, *Uroglena* and *Desmodesmus,* and *Chlamydomonas* did not significantly alter their PUFA proportion with changes in temperature or phosphorus (Fig. 3B, Tables S4 and S7). For the rest of the species, temperature explained on average 25%, whereas phosphorus explained 43% of the observed variance. *Synechococcus* was most affected by temperature (∼90% explained variance), and *Melosira* by phosphorus (∼64% explained variance). Temperature and phosphorus had contrasting effects on PUFA proportion with increases in phosphorus having an overall positive effect regardless of temperature (average Glass $\Delta > 0$, Fig. 3B), whereas increases in temperature alone led to overall decreases in PUFA proportion (average

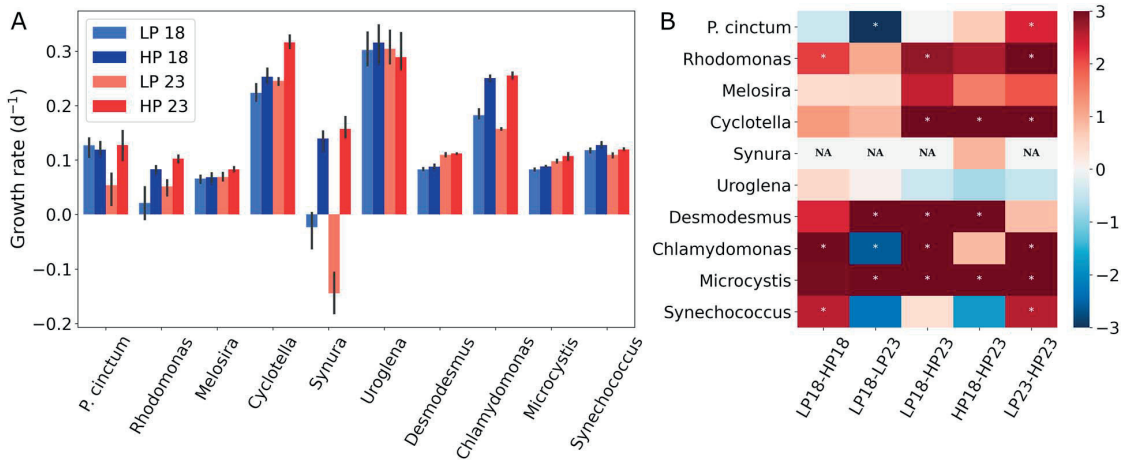

Fig. 1. Growth rate (day^{-1}) per species (**A**) and normalized growth rates (Glass′ Δ) changes between treatments using the first referred treatment as baseline (**B**). Phytoplankton species are representatives of the groups diatoms (*Cyclotella* sp. and *Melosira* sp.), golden algae (*Synura* sp. and *Uroglena* sp.), cyanobacteria (*Microcystis* sp. and *Synechococcus* sp.), green algae (*Chlamydomonas reinhardtii* and *Desmodesmus maximus*), cryptophytes (*Rhodomonas* sp.) and dinoflagellates (*Peridinium cinctum*). Treatment names correspond to culture condition with 18 and 23 denoting temperature in °C and LP and HP denoting phosphorus concentration [0.65 (LP) and 2.58 (HP) μM phosphorus]. ∗ (white marker) denotes statistical difference between treatment comparison in each phytoplankton species.

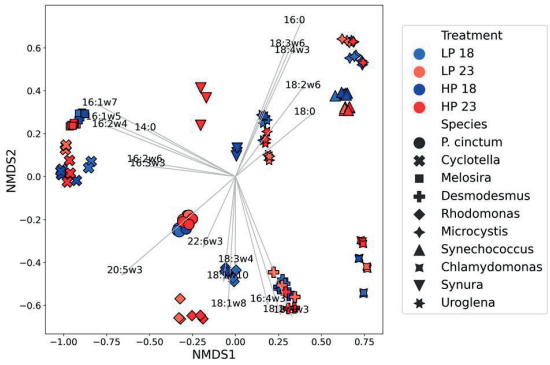

Fig. 2. nMDS of fatty acid compositions. Silver arrows indicate fatty acid direction cosines scaled by the square root of their correlation with the axis. Projected fatty acids (structural formulas) represent saturated (14:0, 16:0 and 18:0), monounsaturated (16:1ω7, 16:1ω5, 18:1ω10, 18:1ω9 and 18:1ω8) and polyunsaturated fatty acids [16:2ω6, 16:2ω4, 16:3ω3, 16:4ω3, 18:2ω6, 18:3ω6, 18:3ω3, 18:3ω4, 18:4ω3, 20:5ω3 (EPA) and 22:6ω3 (DHA)]. For a better visualization of each projected fatty acids, please see Fig. S2. Phytoplankton species are representatives of the groups diatoms (*Cyclotella* sp. and *Melosira* sp.), golden algae (*Synura* sp. and *Uroglena* sp.), cyanobacteria (*Microcystis* sp. and *Synechococcus* sp.), green algae (*Chlamydomonas reinhardtii* and *Desmodesmus maximus*), cryptophytes (*Rhodomonas* sp.) and dinoflagellates (*Peridinium cinctum*). Treatment names correspond to culture condition with 18 and 23 denoting temperature in °C and LP and HP denoting phosphorus concentration [0.65 (LP) and 2.58 (HP) μM phosphorus].

Glass $\Delta < 0$, Fig. 3B). Nevertheless, all treatments presented large size effect differences between species.

In addition to changes in PUFA proportion, we focused on EPA and DHA contents because of their importance for the nutrition of higher trophic levels. Of the studied species that synthesize EPA and DHA, all presented a significant effect of temperature, phosphorus or their interaction (Fig. 4A, Table S5). Both EPA and DHA contents showed large species-specific variation in effect size of temperature and phosphorus (Fig. 4A and B). In terms of EPA content, *P. cinctum* was most affected by changes in phosphorus (69% explained variance; Fig. 4A and B; Table S5), whereas *Uroglena* was most affected by temperature (87% explained variance; Fig. 4A and B; Table S5). EPA content did not present any clear pattern across species to changes in temperature and phosphorus, and the largest average effect size was observed when increasing both temperature and phosphorus (Glass $\Delta \sim 1.2 \pm 2$; Fig. 4B). In the case of DHA content, *Cyclotella* was most affected by changes in phosphorus (61% explained variance; Fig. 4B; Table S5), whereas *Uroglena* was most affected by temperature (81% explained variance; Fig. 4B; Table S5). An increase in phosphorus alone led to positive average effect sizes (Glass $\Delta > 0$), whereas the opposite was seen for increases in temperature alone (Glass $\Delta < 0$). The combined effect of temperature and phosphorus was overall negative (Glass $\Delta \sim -0.17$; Fig. 4B) despite species-specific differences in effect sizes.

The availability of LC-PUFAs in aquatic ecosystems is given by their production, therefore we studied how EPA and DHA daily gain (μg LC-PUFA day^{-1} L^{-1}), a proxy for production, is affected by changes in temperature and phosphorus (Fig. 5A and B). The highest EPA gain

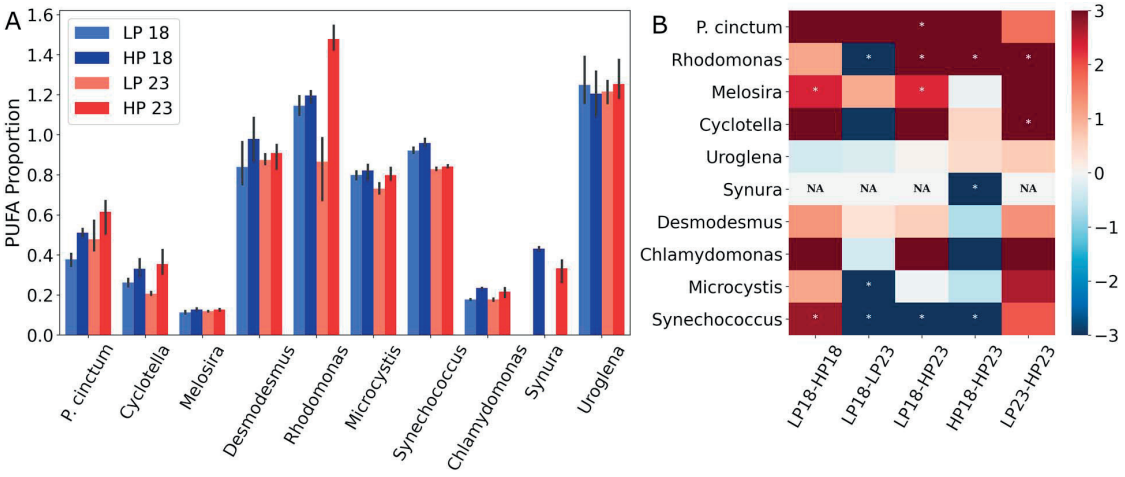

Fig. 3. Proportion of polyunsaturated to mono- and saturated fatty acids (**A**) and their normalized changes (Glass′ Δ) between treatments using the first referred treatment as baseline (**B**). Phytoplankton species are representatives of the groups diatoms (*Cyclotella* sp. and *Melosira* sp.), golden algae (*Synura* sp. and *Uroglena* sp.), cyanobacteria (*Microcystis* sp. and *Synechococcus* sp.), green algae (*Chlamydomonas reinhardtii* and *Desmodesmus maximus*), cryptophytes (*Rhodomonas* sp.), and dinoflagellates (*Peridinium cinctum*). Treatment names correspond to culture condition with 18 and 23 denoting temperature in °C and LP and HP denoting phosphorus concentration [0.65 (LP) and 2.58 (HP) μM phosphorus]. ∗ (white marker) denotes statistical difference between treatment comparison in each phytoplankton species.

was observed in *Cyclotella* (220 ± 71 μg EPA L^{-1} day^{-1}), and the highest DHA gain in *P. cinctum* (179 ± 99 μg EPA l^{-1} day^{-1}). Of our EPA-producing species, *Uroglena's* daily EPA gain was not significantly affected by changes in temperature or phosphorus (ANOVA, Tables S6 and S7). Within the significantly affected species, temperature explained an average of 6.5% of the observed variance in EPA gain, whereas phosphorus explained 53% of the variance (Table S6). DHA daily gain remained unaffected in *Synura*, whereas the rest of the producing species were significantly affected by changes in temperature or phosphorus (ANOVA, Tables S6 and S7). On average, temperature explained 10% of the observed variance, whereas phosphorus explained 52% of the variance observed in DHA gain across significantly affected species (Table S6). Overall, no uniform effect was observed with treatments for either EPA or DHA gain, and effect sizes varied widely between producing species (Fig. 5B). For both EPA and DHA daily gain, the largest average effect sizes were observed when increasing phosphorus at 23°C (Glass $\Delta = 2.57$ and 3.96 for EPA and DHA, respectively; Fig. 5B). *P. cinctum* and *Cyclotella*, the species with the largest EPA and DHA daily gain (respectively) presented opposing effects to the treatments (Fig. 5A and B). *P. cinctum* was most affected by temperature and had a significant interaction term leading to decreasing DHA daily gain when increasing temperature at low phosphorus, whereas *Cyclotella* was most affected by phosphorus and had a significant interaction term leading to decreasing EPA daily gain when increasing phosphorus at 23°C (Fig. 5; Table S6).

DISCUSSION

Climate change is expected to alter northern lakes physical and chemical parameters in variety of ways, including higher temperatures and phosphorus (Jennings *et al.*, 2009; Björnerås *et al.*, 2017). Current projections of LC-PUFAs, in particular EPA and DHA, estimate large decreases in the availability of these fatty acids due to higher temperatures (Hixson and Arts, 2016; Colombo *et al.*, 2019). This study provides evidence challenging the aforementioned assumption while supporting the results of Galloway and Winder (2015) regarding the importance of species composition and lake trophic status on the availability of PUFAs. Our growth rate analysis showed an overall positive effect of temperature and phosphorus across the tested phytoplankton species. The strongest effects were observed when increasing both temperature and phosphorus simultaneously. Nevertheless, large species-specific differences in effect sizes were observed to changes in temperature and phosphorus, highlighting how different life histories and plastic changes can modulate phytoplankton responses. Despite that elucidating the mechanisms behind different phytoplankton responses to the studied treatments was not the objective of this study, we believe that phosphorus absorption kinetics

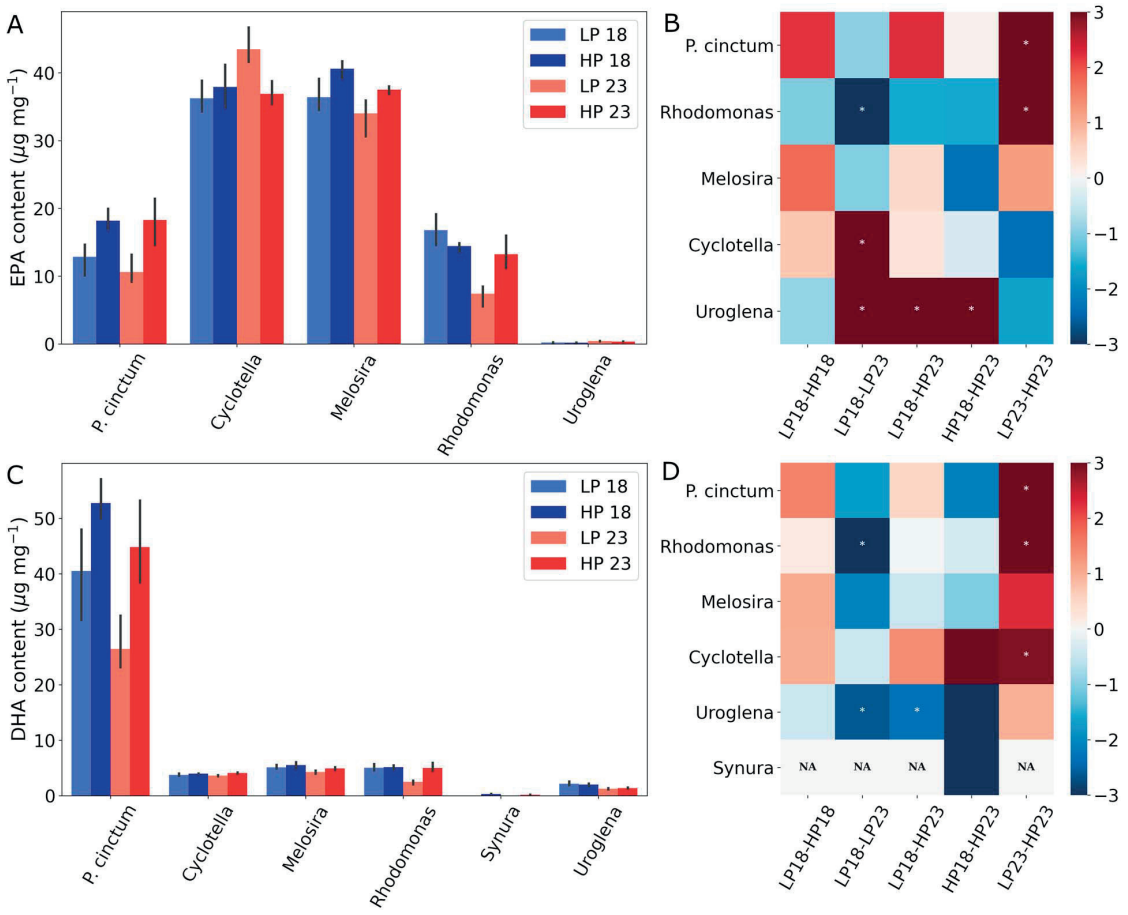

Fig. 4. EPA (**A**) and DHA (**C**) content per mg of dry weight and the normalized EPA (**B**) and DHA (**D**) changes (Glass′ Δ) between treatments using the first referred treatment as baseline. Phytoplankton species are representatives of the groups diatoms (*Cyclotella* sp. and *Melosira* sp.), golden algae (*Synura* sp. and *Uroglena* sp.), cryptophytes (*Rhodomonas* sp.) and dinoflagellates (*Peridinium cinctum*). Treatment names correspond to culture condition with 18 and 23 denoting temperature in °C and LP and HP denoting phosphorus concentration [0.65 (LP) and 2.58 (HP) μM phosphorus]. ∗ (white marker) denotes statistical difference between treatment comparison in each phytoplankton species.

and accumulation strategies (Sommer, 1981) combined with changes in optimal growth temperatures (Singh and Singh, 2015) are the main drivers of the observed results. In northern lakes, increases in total phosphorus and temperature are associated with higher frequencies of cyanobacteria blooms (Keva *et al.*, 2020; Vuorio *et al.*, 2020). Of the two tested cyanobacteria, responses to increases in phosphorus and temperature were contrasting, with only *Microcystis* consistently thriving from such changes. This supports observed trends of increased species-specific cyanobacteria blooms under warm and nutrient-rich environments (O'Neil *et al.*, 2012), highlighting that enhanced growth under those conditions is not an overall property of the phytoplankton group. Although our results suggest substantial increases in phytoplankton biomass with climate change, other physical and chemical changes such as reduced light availability driven by increases in dissolved organic carbon concentrations (browning) can also enforce significant pressures in phytoplankton biomass (Taipale *et al.*, 2016; Sullivan *et al.*, 2021), leading to different phytoplankton responses than the ones observed in this study.

When considering the prospects of PUFAs in aquatic ecosystems, temperature increase is associated with PUFA decrease to maintain membrane homeostasis (Sinensky, 1974). In our results, neither temperature nor phosphorus had a generalized effect in phytoplankton fatty acid profiles or the PUFA proportion. At a species level, temperature and phosphorus did affect PUFAs, but variability in the directionality of change and effect size was high. Among the studied species, an increase in phosphorus had an overall positive effect on PUFA proportion. Increases

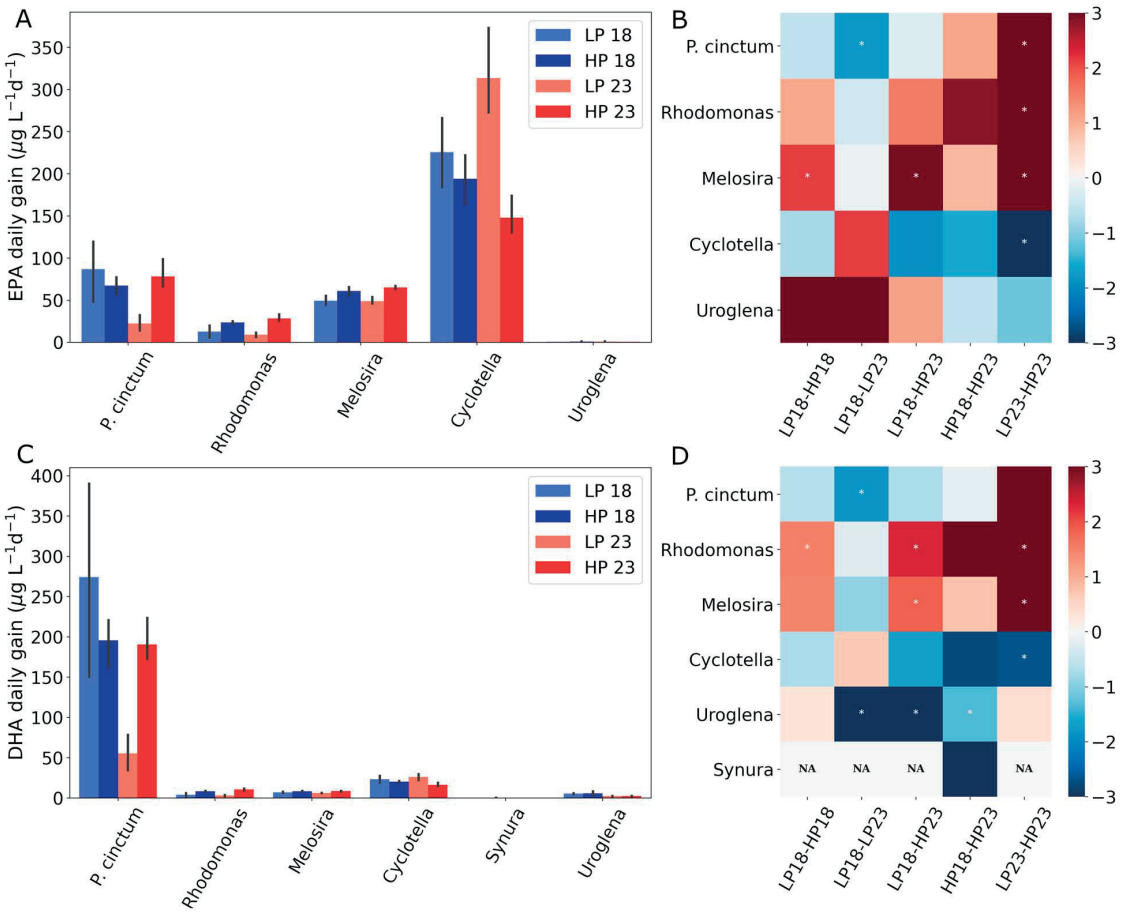

Fig. 5. EPA (**A**) and DHA (**C**) production measured as daily gain (μg L^{-1} day^{-1}) and the normalized EPA (**B**) and DHA (**D**) production changes (Glass′ Δ) between treatments using the first referred treatment as baseline. Phytoplankton species are representatives of the groups diatoms (*Cyclotella* sp. and *Melosira* sp.), golden algae (*Synura* sp. and *Uroglena* sp.), cryptophytes (*Rhodomonas* sp.) and dinoflagellates (*Peridinium cinctum*). Treatment names correspond to culture condition with 18 and 23 denoting temperature in °C and LP and HP denoting phosphorus concentration [0.65 (LP) and 2.58 (HP) μM phosphorus]. ∗ (white marker) denotes statistical difference between treatment comparison in each phytoplankton species.

in temperature alone led to an overall decrease in PUFA proportion regardless of initial phosphorus concentration. Altogether, these results suggest that concomitant increases in phosphorus could hinder the decrease in PUFA proportion due to higher temperatures, and that other physical and chemical changes associated with climate change could play a more significant role than previously thought. For example, light availability can modulate cellular levels of PUFAs (Valentine and Valentine, 2004; Wacker *et al.*, 2016) and phytoplankton communities (Bergström *et al.*, 2003; Deininger *et al.*, 2017). Although browning has not been observed to alter PUFA contents in a phytoplankton species common to high dissolved organic carbon lakes (Calderini *et al.*, 2022), overall phytoplankton PUFAs will depend on the interaction between eutrophication, warming and browning. Our results on LC-PUFAs EPA and DHA contents show variable responses of individual phytoplankton species to increases in temperature and phosphorus. Contrary to previous studies (Hixson and Arts, 2016; Colombo *et al.*, 2019), no clear pattern of EPA content to increase in temperature or phosphorus was seen across the studied species. In the case of DHA, a general reduction of DHA content was seen with temperature increases regardless of trophic state, partially agreeing with previous estimates (Colombo *et al.*, 2019). Nevertheless, large species-specific differences in effect size were observed, with some species presenting an increase in DHA content with increases in phosphorus suggesting that concomitant increases in temperature and phosphorus could modulate the negative effect of temperature as we hypothesized. As an extension of these results, it is possible that lakes exhibiting a

pattern of warming coupled with declines in nutrient levels (Isles *et al.*, 2018; Isles *et al.*, 2023) could experience reductions of PUFA (particularly DHA) availability.

Although LC-PUFA content is the unit commonly used to study the nutritional quality of seston in lakes, we looked at LC-PUFA production (studied as daily gain) to account for the effect of changes in cell density observed with our treatments. The production of EPA and DHA shows large species-specific differences in directionality and effect size in response to changes in phosphorus and temperature. Of the studied phytoplankton groups, diatoms are considered key EPA producers in aquatic ecosystems, whereas DHA production is associated with dinoflagellates and golden algae (Ahlgren *et al.*, 1992; Galloway and Winder, 2015; Taipale *et al.*, 2016; Jónasdóttir, 2019). Within the studied diatoms, we did not see consistency in their response to changes in temperature and phosphorus, with *Cyclotella* (highest EPA production) showing an overall negative effect to increases in both temperature and phosphorus, whereas *Melosira* showed a substantial increase in EPA production with an increase in phosphorus. Of the studied dinoflagellates and golden algae, both *P. cinctum* (highest DHA daily gain) and *Uroglena* showed an overall decrease of DHA daily gain with increases in temperature. Nevertheless, effect sizes varied largely between these species and within treatments. In summary, the variability observed in effect sizes and directionality of response to warming and phosphorus concentrations across the studied species suggest that species composition will be the determining factor in the availability of PUFAs and LC-PUFAs in lakes as proposed by Galloway and Winder (2015). In addition, processes that increase nutrients (e.g. eutrophication, browning) could play an important role in modulating the effects associated with warming. Altogether, cold water oligotrophic lakes, which commonly present large shares of diatoms in their phytoplankton communities (Taipale *et al.*, 2016; Keva *et al.*, 2020), could present notable fluctuations in EPA production given the observed species-specific responses to environmental change. Meanwhile, DHA production could be more affected in eutrophic lakes with large amounts of dinoflagellates (Taipale *et al.*, 2019). Variations in EPA and DHA production, as a result of species-specific responses of diatoms and dinoflagellates, could potentially alter zooplankton communities due to the high requirements of cladocerans for EPA (Von Elert, 2002) and copepods for DHA (Von Elert and Stampfl, 2000).

Importantly, we did not see a correlation between content and production results for EPA or DHA, showing that these units point to different aspects of phytoplankton LC-PUFAs availability for consumers. Fatty acid content does not account for changes in cell densities when studying the effects of environmental change, hence higher phytoplankton growth can compensate for low fatty acid content. This is especially relevant to consider when extrapolating fatty acid content results to make predictions about how climate change will affect the availability of LC-PUFAs.

CONCLUSIONS

This study shows that trophic state, as well as phosphorus dynamics, will play a role in the availability of PUFAs and LC-PUFAs in lakes. Both warming and phosphorus will influence PUFAs differently, with temperature driving decreases while phosphorus increases in PUFA availability. EPA and DHA producing species respond differently to increases in temperature and nutrients both in terms of content and daily gain of these fatty acids. Therefore, temperature, phosphorus and phytoplankton composition in lakes will all determine the effects of climate change on the availability of the physiologically essential EPA and DHA.

DATA AVAILABILITY

All data as exportable files and data analysis scripts necessary to replicate the results presented in this study are available at jyx data storage service (10.17011/jyx/dataset/86595). A comprehensive description of the content of each file can be found in the same DOI under the name "Metadata_file."

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SUPPLEMENTARY DATA

Supplementary data can be found at *Journal of Plankton Research* online.

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III

EUTROPHICATION EFFECT ON PRODUCTION AND TRANSFER OF OMEGA-3 FATTY ACIDS IN BOREAL LAKE FOOD WEBS

by

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Eutrophication effect on production and transfer of omega-3 fatty acids in boreal lake food webs

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Abstract

Eutrophication, i.e. increasing level of nutrients and primary production, is a central environmental change of lakes globally with wide effects on food webs. However, how eutrophication affects the synthesis of physiologically essential biomolecules (omega-3 fatty acids) and their transfer to higher trophic levels at the whole food web level is not well understood. We assessed food web (phytoplankton, zooplankton, and fish) biomass, community structure and fatty acid content (eicosapentaenoic acid [EPA], and docosahexaenoic acid [DHA]), together with fatty acid specific primary production in 12 Finnish boreal lakes covering the total nutrient gradient from oligotrophic to highly eutrophic lakes (4–140 µg TP l^{-1}; 413–1,814 µg TN l^{-1}). Production was measured as the incorporation of ^{13}C-$NaHCO_3$ into phytoplankton fatty acids and differentiated into volumetric production (production per litre of water) and productivity (production per phytoplankton biomass). Increases in nutrients led to higher biomass of phytoplankton, zooplankton and fish communities while also affecting community composition. Eutrophication negatively influenced the contribution of phytoplankton biomass preferentially grazed by zooplankton (<35 µm). Total volumetric production saturated at high phytoplankton biomass while EPA volumetric production presented a logarithmic relationship with nutrient increase. Meanwhile, total and EPA productivity had unimodal responses to this change in nutrients. DHA volumetric production and productivity presented large variation with increases in total phosphorus, but a unimodal model best described DHA changes with eutrophication. Results showed that eutrophication impaired the transfer of EPA and DHA into zooplankton and fish, showing a clear negative impact in some species (e.g. perch) while having no effect in other species (e.g. roach, ruffe). Results show non-linear trends in fatty acid production and productivity peaking at nutrient concentrations 22−35 µg l^{-1} TP followed by a gradual decrease.

Keywords

Boreal lakes, DHA, EPA, eutrophication, food webs, production, productivity.

1. Introduction

Primary production is the fundamental process that fuels food webs (Underwood & Kromkamp 1999). In aquatic environments, the principal primary producers providing energy and nutrients to secondary producers are pelagic phytoplankton, benthic periphyton, and macrophytes (McLusky & Elliott 2004). The pelagic and benthic primary production ratio varies largely across ecosystems given the influence of morphological characteristics like depth, and environmental conditions such as light, temperature and nutrient supply (e.g., Hauxwell & Valiela 2004; Hayden et al. 2019). Eutrophication, commonly described as an increase in nutrients and especially phosphorus (Hasler 1947; Schindler 1974), is one of the major challenges faced by freshwater ecosystems across the globe. In addition to focal point discharge of nutrients from sewage and industrial sources, more complex factors such as diffuse pollution from agriculture and forestry enforced by climate change increase nutrient concentrations in water bodies (Jennings et al., 2009; Keatley et al., 2011). Climate change has already increased precipitation in the boreal region, facilitating the run-off of nutrients from catchment areas (de Wit et al., 2016; Ruosteenoja et al., 2016; IPCC, 2014). Nutrient run-off is particularly influential in water bodies with agricultural and peatland-forestry dominated boreal catchments (Räike et al., 2020; Finér et al., 2021), raising concerns about the acceleration of

eutrophication with climate change and its effects on aquatic primary production (Jeppesen et al., 2012; IPCC, 2014).

Eutrophication has been shown to boost pelagic phytoplankton growth, supressing benthic primary production by reducing light, up-taking available nutrients and promoting hypoxia at increasing depths (e.g., Valiela et al., 1997; McGlathery et al., 2007; Qin & Shen, 2019). Such effects of eutrophication alter the balance of benthic and pelagic primary production, increasing reliance of whole aquatic food webs on the latter (Hayden et al., 2019). Complementary to changes in primary production, eutrophication has large effects on aquatic food web community structure at different trophic levels (Vollenweider et al., 1974; Jeppesen et al., 2000; Thackeray et al., 2008; Hayden et al., 2017; Keva et al., 2021). Changes in phytoplankton community composition can greatly affect the availability of micronutrients such as omega-3 fatty acids (FAs) since not all polyunsaturated FAs (PUFAs) are equally synthesised by different phytoplankton taxa (Ahlgren et al., 1992; Lang et al., 2011, Taipale et al., 2013). Among the high nutritional value omega-3 polyunsaturated FAs, long-chain eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3) are essential for the development and reproduction of consumers (Arts, 2001; Parrish, 2009). Diatoms, dinoflagellates, golden algae and cryptophytes are able to produce EPA and DHA efficiently, whereas cyanobacteria and green algae may contain only trace amounts of these FAs (Müller-Navarra et al., 2004; Taipale et al., 2016b). As observed across nutrient gradients (O'Neil et al., 2012; Rigosi et al., 2014; Keva et al., 2021; Taipale et al., 2022a), eutrophication shifts phytoplankton communities from high to low EPA and DHA. This change in phytoplankton is expected to have putative cascading effects on the EPA and DHA contents of higher trophic levels due to the high metabolic cost of bioconversion or de-novo synthesis (Müller-Navarra et al., 2004; Hixson & Arts, 2016; Taipale et al., 2016b; Lau et al., 2021).

Much of previous research on lake food web nutritional quality (EPA and DHA levels) has focused on phytoplankton-zooplankton (Senar et al., 2019; Lau et al., 2021) or phytoplankton-fish interfaces (Taipale et al., 2016b; Marques et al., 2020; Gomes et al., 2021), but studies looking at several food web components are still relatively scarce (Strandberg et al., 2015; Kainz et al., 2017; Keva et al., 2021). Recent comparisons of food web components' nutritional quality in different types of lakes suggested efficient trophic upgrading of PUFAs when phytoplankton nutritional quality was low (Kainz et al., 2017; Keva et al., 2021; Taipale et al., 2022a). These results challenge views of direct PUFA trophic transfer across trophic levels (e.g., Strandberg et al., 2015; Kainz et al., 2017). However, the utilization of different methodologies and units of measurement could potentially contribute to the observed mismatch between theory and current literature (Taipale et al., 2016b; Keva et al., 2021; Gomes et al., 2021; Lau et al., 2021). For example, phytoplankton EPA and DHA content per unit of carbon have shown a steep linear decrease with increases in nutrients (Müller-Navarra et al., 2004; Trommer et al., 2019). Nevertheless, seston EPA and DHA contents (μg mg^{-1} dry weight) have suggested high variation in eutrophic lakes whereas strong decrease in EPA and DHA can be seen only in hyper-eutrophic lakes with frequent cyanobacteria blooms (Müller-Navarra et al., 2004; Taipale et al., 2019). Meanwhile, volumetric EPA and DHA concentrations (μg FA l^{-1} water) tend to be highest in eutrophic lakes (Taipale et al., 2016b; Strandberg et al., 2022). These different units point to the different aspects of phytoplankton nutritional quality and potential transfer to upper trophic levels. High volumetric concentrations of PUFAs can indicate high phytoplankton biomass and hence large amounts of nutritious

prey for zooplankton. On the other hand, PUFA contents indicate the average quality of the phytoplankton biomass but no information on prey quantity for zooplankton is obtained. Therefore, unravelling the dynamics of PUFA transfer across trophic levels requires careful consideration of methodology and units selected, as well as a holistic approach of FA transfer across food webs.

Altogether, current literature point to a knowledge gap in the understanding of the effects of eutrophication on PUFA dynamics across aquatic food webs and raise the question of what are the key factors explaining the mismatch between phytoplankton and higher trophic levels nutritional quality. Among the putative factors, increasing knowledge on PUFA bioconversion by invertebrates and fish (Kabeya et al., 2018; Ishikawa et al., 2019; Boyen et al., 2023) suggest that such mechanisms might be more prevalent and efficient than previously estimated. Nevertheless, it is also possible that phytoplankton PUFA content may not be an ideal unit to study FA quality at higher trophic levels due to the high top-down regulation of phytoplankton biomasses. Zooplankton grazing has been suggested to be selective for small phytoplankton sizes (Porter, 1973; Knisely & Geller, 1986; Heathcote et al., 2016; Lürling, 2021), and thus the effects of grazing can rapidly shape phytoplankton communities. The nutritional value of the remaining phytoplankton in the water column could be overrepresented by low quality non-edible biomass. Moreover, zooplankton has the capacity to retain certain PUFAs (Taipale et al., 2011; Hartwich et al., 2012), increasing the mismatch between zooplankton and phytoplankton PUFA contents. Therefore, short-time measurements of phytoplankton FA production could reveal how high value PUFAs are produced and available for higher trophic levels before zooplankton predation. Previously, phytoplankton production has been measured with either bulk ^{14}C incorporation methods or oxygen exchange techniques (Underwood & Kromkamp, 1999). Despite the insights given by these techniques, their resolution is low, meaning that no information is obtained about where the newly fixed carbon is metabolically channelized. Using ^{13}C-labeling and compound-specific isotopes could be more reliable tools to measure primary production of individual biomolecules (Dijkman et al., 2009; Gladyshev et al., 2012; Middelburg 2014; Lammers et al., 2016). The use of ^{13}C-labeling and FA-specific isotopes could help identify the production of high quality PUFAs that are to be consumed by selective grazers. In addition, the impact of nutrient availability on PUFA production could shed light into how eutrophication affects phytoplankton efficiency to produce specific nutrients for higher trophic levels (Taipale et al., 2016b).

In this study, 12 boreal lakes across a total phosphorus (TP) and nitrogen (TN) gradient were sampled to study how eutrophication affects pelagic phytoplankton, zooplankton and fish relative biomass, community composition as well as EPA and DHA contents. In addition, chamber incubations were conducted to measure how phytoplankton FA production is affected by eutrophication and how this impacts the transfer of high value PUFAs across food webs. For this purpose, we used ^{13}C labelled $NaHCO_3$ as a substrate to follow the incorporation of inorganic carbon into phytoplankton FAs. Then, Compound Specific Isotope Analysis (CSIA) of individual FAs was employed to calculate a proxy for total primary production, as well as specific EPA and DHA productions. We propose the distinction between two units: volumetric production, as a measure of production per litre of water, and productivity, as a measure of production per phytoplankton biomass. We consider that this distinction provides alternative perspectives on the interaction between phytoplankton production and zooplankton. We hypothesise that pelagic phytoplankton biomass increases linearly with TP, as previously observed (e.g. Schindler,

1977; Keva et al., 2021), but volumetric production levels off at high phytoplankton cell densities given competition for nutrients and light (Han et al., 2000). In oligotrophic lakes, we hypothesise that productivity (total and PUFA specific) is limited by nutrients, hence increases in nutrients drive increases in fixation of inorganic carbon per cell (Han et al., 2000). Nevertheless, at higher nutrient concentrations, high phytoplankton cell densities increase competition for light leading to lower productivity and an overall unimodal relationship with TP (Persson et al., 2007; Taipale et al., 2019). The transition in phytoplankton communities from high to low EPA and DHA observed with eutrophication (O'Neil et al., 2012; Rigosi et al., 2014; Keva et al., 2021) is likely to further decrease the productivity of these PUFAs at high nutrient levels. We expect that the patterns of EPA and DHA productivity across the TP gradient match the contents of these PUFAs in short longevity cladoceran zooplankton and pelagic fish, while divergent patterns are expected in long longevity copepods and benthic or generalist fish.

2. Materials and methods

2.1. Study sites and field sampling

We selected 12 boreal lakes located in middle and southern Finland spanning a marked phosphorus and nitrogen gradient (total phosphorus; TP: 4–140 µg l^{-1}, total nitrogen; TN: 413–1,814 µg l^{-1}; Fig. 1; Table 1) as a proxy to study the effects of eutrophication. For a simplified view of the methods used in this study please see Fig. 2. Lakes were selected based on their long-term monitoring data on water TP and TN concentrations measured between July to August during the years 2001-2020. (Fig. S1). Long-term water chemistry parameters were retrieved from the Finnish Environment Institute (Syke) (HERTTA-database) while the presented maps (Fig. 1) were obtained from Syke and the National Land Survey of Finland. Dissolved organic carbon concentration during the study year varied between 5.9–19 µg C l^{-1} among the studied lakes (Table 1). Percentage of agriculture in catchment area varied between 0–18.5% and closed forest coverage varied between 43–90% in the studied lakes (Table S1). Catchment properties were obtained using the VALUE-tool (Syke) combining catchment, the CORINE Land Cover inventory and open map data. The selected lakes were sampled once during the summer season (July) of 2021 in randomised order with a difference of 15 days between the first and last sampled lake. Surface water temperatures varied between 17.6–26.8 °C between lakes. Water samples for analysis of seston FA and production measurements were collected from the photic zone (0.3–9.0 m depending on the lake; Table 1) corresponding to twice the measured Secchi depth. For FA analysis, seston samples were filtered through 3.0 µm cellulose nitrate membranes (Whatman, GE Healthcare) and kept at −80°C. Experimental production measurements are described later in this section. Dissolved inorganic carbon concentration was determined with an Agilent 7890B GC (Agilent) as previously described (Taipale et al., 2022b). Samples for chlorophyll-a concentration determination were filtered with GF/C filters (Whatman, nominal pore size 1.2µm) and analysed with a Shimadzu UV-1800 spectrophotometer after 94% ethanol (wt%) extraction (at 75 °C for 5 min) (Keskitalo & Salonen 1994). DOC concentration was measured from HCl-acidified samples (final pH = 2) using a carbon and nitrogen analyser (TOC-L, Shimadzu). The rest of the water chemistry analyses presented in Table 1 were conducted by Lammi Biological Station laboratory (University of Helsinki, Finland). Zooplankton samples were collected by multiple hauling in the water column from lake bottom to surface with plankton nets (100, 250 and 500 µm mesh sizes) to obtain sufficient zooplankton amounts for subsequent FA analyses. Samples were then kept at

6°C and sorted to genus level. For subsequent analysis, distinction was made between the obtained Cladocera taxa based on feeding strategy with *Bosmina* sp., *Daphnia* sp. and *Holopedium gibberum* categorized as herbivorous Cladocera and *Leptodora kindtii* as predatory Cladocera. Copepod genera were classified into the orders Calanoida (*Eudiaptomus*, *Heterocope*, and *Limnocalanus*) and Cyclopoida. Fish were collected from July-August 2021 sampling littoral, pelagic, and profundal habitats using Nordic gillnets as described below (section Community composition and biomass). Fish were immediately removed from the nets and euthanized by cerebral concussion and placed on ice. All fish were identified and measured for total length (accuracy 1 mm) and weight (0.1 g). The most recently entangled fish (with red gills) were selected for fatty acid analyses including perch (*Perca fluviatilis*), ruffe (*Gymnocephalus cernua*), roach (*Rutilus rutilus*) and smelt (*Osmerus eperlanus*).

2.2. Community composition and biomass

Phytoplankton samples were taken from pooled water corresponding to the photic zone collected with a 5 l water sampler (Limnos Ltd). Samples were immediately stored in acid Lugol solution (1 ml per 200 ml). Phytoplankton analyses were performed under an inverted microscope by applying the Utermöhl technique (Utermöhl, 1958, SFS-EN 15204). Phytoplankton were identified to species level when possible. Biovolumes were converted to fresh weight biomass estimations using taxa morphology-specific geometric formulas (Hillebrand et al., 1999) and assuming phytoplankton density to equal that of water. Biomass values were further converted to carbon contents using carbon-mass ratios (Menden-Deuer & Lessard, 2000). Calculated class-specific percentage of phytoplankton was obtained utilizing carbon biovolume (µg C l^{-1}) data. Size classification of phytoplankton into edible (<35 µm) and non-edible (>35 µm) was done according to the widest dimension based on size preference of food particles for zooplankton (Watson & McCauley, 1988; Heathcote et al., 2016). For example, if the cell presented spines or bristles extending further than 35 µm, then they were classified non-edible. Colonies and filament forming species were also classified as non-edible despite the size of individual cells. Long-term July-August phytoplankton biomass data (2001-2020) of ten of the studied lakes was obtained from the National Finnish Phytoplankton Monitoring Database maintained by the Syke.

Quantitative pelagic zooplankton samples were taken from pooled water corresponding to the whole water column collected with a 7.1 l water tube (Limnos Ltd). Samples were filtered through a 50 µm mesh net and preserved in 70% ethanol. Zooplankton were enumerated, body length and width measured under an inverted microscope, and identified to the species level. Zooplankton are presented as larger groups as follows: herbivorous Cladocera (*Alona*, *Bosmina*, *Ceriodaphnia*, *Chydorus*, *Daphnia*, *Diaphanosoma*, *Holopedium*, *Limnosida*), predatory Cladocera (*Leptodora*), Cyclopoida (*Cyclopoida nauplius*, *Cyclopoida copepodite*, *Diacyclops*, *Megacyclops*, *Mesocyclops*, *Thermocyclops*), Calanoida (*Calanoida nauplius*, *Calanoida copepodite*, *Eudiaptomus graciloides*, *Heterocope Limnocalanus*), Rotifera (*Anuraeopsis*, *Ascomorpha*, *Asplanchna*, *Brachionus*, *Chromogaster*, *Collotheca*, *Colyrella*, *Conochilus*, *Conochiloides*, *Filinia longiseta*, *Gastropus*, *Kellicottia*, *Keratella*, *Notholca*, *Ploesoma*, *Bipalpus*, *Polyarthra*, *Pompholyx*, *Synchaeta*, *Trichocerca*), and *Chaoborus*. From each species, first 30 individuals were measured to estimate the carbon biomass using species-specific carbon regressions (Bottrell et al., 1976; Vasama & Kankaala, 1990; Luokkanen,

1995). The overall zooplankton community composition was calculated as taxon-specific percentage from carbon biomass (mg C l^{-1}).

Fish community composition and biomass per unit of effort (BPUE) data was obtained from the Natural Resources Institute Finland (LUKE). Data represents the summer 2021 sampling season for 11 of the lakes sampled in our study, while Lake Enäjärvi corresponds to 2019 (latest sampling in database). Fish samples were collected according to the European Standard (EN 14757:2005; CEN 2005) using Nordic multi-mesh gillnets with 30 m length x 1.5 m height (12 x 2.5 m wide panels, mesh sizes: 5-55 mm). Number and position of gillnets depended on lake morphological characteristics, but generallypelagic, littoral and profundal habitat types were sampled (CEN 2005). Gill nets were set in the evening and collected during the following morning (approx. 12 hr soaking time) at a minimum of two sampling nights per lake. For fish community composition analysis, identified species were grouped as percids (perch, pikeperch, *Sander lucioperca* and ruffe), cyprinids (minnow, *Phoxinus phoxinus*, roach, white bream, *Blicca bjoerkna*, common bream *Abramis brama* and blue bream, *Ballerus ballerus*, common bleak *Alburnus alburnus*, crucian carp, *Carassius carassius,* ide, *Leuciscus idus*, rudd, *Scardinius erythrophthalmus*, and tench, *Tinca tinca*), salmonids (landlocked salmon, *Salmo salar* m. sebago, vendace, *Coregonus albula*, European whitefish; *C. lavaretus*) and others (smelt, pike, *Esox lucius*, burbot, *Lota lota*, common bullhead, *Cottus gobio*).

2.3. Lipid extraction and fatty acid analysis

Total lipids were extracted from seston (filtered samples), zooplankton and fish (dorsal muscle samples). In total, 24 seston samples (two replicates for each lake), 41 zooplankton samples (21 cladocerans, 20 copepods; two replicates of each) and 331 fish samples (44 smelt, 57 roach, 175 perch and 55 ruffe) were analysed across the 12 studied lakes. Total lipids were extracted with 3.75 ml chloroform/methanol/water (4:2:1) (Folch et al., 1957) using sonication (10 min). After phase separation, solvents were evaporated at 50°C under a nitrogen stream. The sample was evaporated and dissolved in 1 ml toluene, and FAs were transesterified overnight (50°C) using methanolic H_2SO_4 (1%, v/v). FA methyl esters were analysed with a gas chromatograph equipped with a mass detector (GC–MS; Shimadzu Ultra) using a DB-FastFAME column (30 m × 0.25 mm × 0.25 μm; Agilent) using a split injector mode. The temperature program: 60 °C was maintained for 1 min, and then the temperature was increased at 40 °C min^{-1} to 165 °C and held for 1 min, temperature was then increased by 4 °C min^{-1} to 230 °C and held for 4.5 min. The column flow was set at 0.99 ml min^{-1}. Four quantification calibration curves (concentrations 50, 100, 250 and 500 ng μl^{-1}) were prepared with FA standard GLC reference standard 556 C (Nu-Chek Prep, Elysian). FAs in sample spectrums were identified using retention times together with specific ions. Quantification of FAs was based on detector responses, the peak areas were integrated using GCsolution software (version 2.41.00, Shimadzu) and sample FAs concentrations were obtained by interpolation in the reference standard calibration curve. Recovery percentages (>70% for all samples) were calculated using an internal standard added to each sample before lipid extraction (free FA C23:0; Larodan). Recovery percentages were used to correct sample FA concentrations. At the zooplankton level, since EPA and DHA are distinctively accumulated in cladocerans and copepods, EPA content dynamics with TP was studied from cladocerans while the same was done with copepods and DHA.

2.4. Production measurements and compound specific stable isotope analysis

To examine the relationship between eutrophication and phytoplankton carbon fixation, the incorporation of $\delta^{13}C$ from labelled $NaHCO_3$ into phytoplankton FAs was a proxy for primary production. Here, 99% ^{13}C - $NaHCO_3$ (Sigma-Aldrich) was added to 160−1000 ml of 2xSecchi depth water to a final amount corresponding to 4% of the total dissolved inorganic carbon measured in each lake. Differences in volume were given by differences in the amount of particulate matter present in the water. Incubation time was 2 hr at constant light (70−96 μmol quanta m^{-2} s^{-1}) at 21°C. After incubation, samples were filtered with GF/F filters (Whatman) and stored at −80 °C. Duplicate samples for each lake were obtained. Lipids were extracted and FAs were methylated and quantified as described in the lipid extraction and fatty acid analysis section before being analysed for compound specific stable isotopes. In addition, duplicates of non-labelled samples of the same 2xSecchi depth water were filtered, extracted, and analysed together with labelled samples. The $\delta^{13}C$ values of FAs were determined using a GC-MS (Agilent 7890B GC, Agilent 5977B MS) connected to an isotope ratio mass spectrometer (Isoprime precisION, Elementar). FAs were first separated using a 30 m ZB-23 column (0.25 mm x 0.15 mm; Phenomenex) and then oxidized to carbon dioxide in an oxidation reactor at a temperature of 940°C with the reduction reactor kept at 630°C. The separation temperature for GC: 50 °C was held for 1 min, and then the temperature was increased at 10 °C min^{-1} to 130 °C, temperature was then increased by 7 °C min^{-1} to 180 °C and then by 1°C min^{-1} to 210°C and held for 3 min. Finally, temperature was raised to 260°C at the rate of 10°C min^{-1}. FA internal standard (FREE 23:0) was used for drift and linear correction. The calculated precision for the standard used was ± 0.4‰, and the accuracy was ± 0.3‰. The $\delta^{13}C$ value of methanol (derivatization reagent) was obtained with EA-SIRMS system (Finnigan DELTAplus Advantage, Thermo Fisher Scientific) for correction of individual FAs. The $\delta^{13}C$ values of individual FAs were manually calculated using individual background values and corrected for the carbon atom in the methyl group that was added during derivatization as described by Dijkman et al., (2009). In total, we were able to identify and quantify the $\delta^{13}C$ values of 13 FAs (14:0, 15:0, 16:0, 16:1ω-7c, 18:0, 18:1ω-9, 18:1ω-7, 18:3ω-6, 18:3ω-3, 18:4ω-3, 20:4ω-6, 20:5ω-3, 22:6ω-3).

2.5. Data analysis

Linear regression to describe relationships between the studied biomass or proportion of edible phytoplankton and TP can be found in Table S2. In addition to linear regression, we tested Log-Log linear regression in which both the predictor and response variable are transformed using natural logarithm. Principal component analysis (PCA) was carried out using contribution data of phytoplankton, zooplankton and fish communities using the library sklearn for PYTHON programming language. Compound specific stable isotope data ($\delta^{13}C$) from labelled and non-labelled FA samples was first converted to ratio abundance of the heavy isotope (^{13}F) according to the following formula (Fry 2006):

$$^{13}F = (\delta^{13}C_{FA} + 1000) / (\delta^{13}C_{FA} + 1000 + (1000/ R_s))$$

Where $\delta^{13}C_{FA}$ is the $\delta^{13}C$ value of each FA, and R_s corresponds to the Vienna Pee Dee Belemnite (VPDB) standard $^{13}C/^{12}C$ value = 0.01118 (Ding et al., 2001). Based on our results, we established two units: (1) Volumetric production, describing phytoplankton FA production $litre^{-1}$ $hour^{-1}$ and (2) Productivity, describing production seston dry $weight^{-1}$ $hour^{-1}$. The corresponding values of each individual FA in each lake were obtained as follows:

$$\text{Volumetric production}_{FA}\ (\text{pg l}^{-1}\ \text{hr}^{-1}) = ({}^{13}F_E - {}^{13}F_N) \times C_{FA} / \text{Time}_{inc} \times 1000$$

Where ${}^{13}F_E$ is the abundance ratio of ^{13}C of FA in the enriched sample, ${}^{13}F_N$ is the mean abundance ratio of ^{13}C of the same FA in the non-enriched samples, C_{FA} is the carbon concentration of the FA in ng l^{-1} (quantified by GC-MS, as described in lipid extraction and fatty acid analysis) in the enriched sample, and Time_{inc} is the incubation time. Productivity_{FA} followed the same formula, but C_{FA} corresponded to carbon concentration of the FA in ng mg^{-1} of dry weight. Since we distinguished $\delta^{13}C$ incorporation into different FAs, Total Volumetric Production (TVP) and Total Productivity were derived from the added production or productivity value of all individual FAs, respectively.

Linear and non-linear models fitted to describe relationships between our production, productivity, EPA and DHA content of zooplankton and fish can be found in Table S3 and Table S4. For all models tested in this study, model selection was based on Akaike Information Criterion (AIC). Nevertheless, residual plots were first checked to verify that the model described well the mean structure of the data. If residuals presented a clear trend (not randomly distributed) in the residual vs fitted plots, the model was automatically discarded. Additionally to AIC values, p-value of model coefficients, Root Mean Square Error (RMSE), confidence interval of coefficient estimates are presented for all models. For non-linear models, 95% confidence intervals of model coefficients were obtained by non-parametric bootstrapping. Rejection of null hypothesis for non-linear model coefficients was done by looking at p-values, confidence intervals, and normality (visual checking) of frequency distribution of coefficient values obtained by nonparametric bootstrapping (1000 iterations). If frequency distribution was classified as skewed, the null hypothesis was not rejected due to the uncertainty in the confidence interval of the model parameter. Values of r^2 were only presented in linear models due to their limitation in non-linear models (Spiess et al., 2010). The non-linear models presented in this study correspond to the density dependent models proposed by Beverton-Holt (1957) and Ricker (1954).

Beverton-Holt (1957) model:

$$y = ax / (1 + bx)$$

Where y represents the response variable (indicated in each studied case) that approaches an asymptotic value as x increases, x the independent variable (total phosphorus or phytoplankton biomass), a is the density-independent parameter of which the value is the slope of the model near x = 0, and b is the density-dependent parameter. If density dependence does not exist, then b = 0. Maximal asymptotic value of the response variable was calculated as a/b. Based on this, the value of x that produces half of the asymptotic value was calculated using the model.

Ricker (1954) model:

$$y = ax\ e^{(-ax\ /\ Pp\ e)}$$

Where y represents the response variable (indicated in each studied case) that decreases past a threshold in x, x the independent variable (total phosphorus in the presented plots), a is the density-independent parameter, Pp is the peak production or productivity, and e represents an exponential function. The value of the independent variable x that produced the peak production was then calculated as:

$$x = Pp\ e^{(1)} / a$$

Model fitting, hypothesis testing and confidence intervals for model coefficients of these non-linear models were done with the R packages nlstools, FSA and FSAdata. Non-linear models, as well as Log-Log linear models, were fitted using natural logarithm transformed data, hence their AIC value were corrected for comparison with linear models as previously described (Akaike, 1978). All presented models that were fitted on logarithm transformed data were back transformed to their original units for plotting and for RMSE calculation. An alpha level of 0.05 was used in each statistical analysis and tests were conducted using RStudio version 4.0.5 with the default base package (R Core Team, 2017).

3. Results

3.1. Food web biomass and community structure

The biomass of each of the studied lake food web components increased along the TP gradient with changes in community composition due to a more pronounced increase in fish and phytoplankton than in zooplankton (Fig. 3; Fig. S2). At the base of the food web, phytoplankton biomass increased 1.14% for every 1% increase in TP ($\ln(y)=2.44 + 1.14\ln(x)$; $r^2=0.78$, $p<0.01$; Fig. 3A; Table S2). This increase in phytoplankton biomass accompanying increases in TP has been observed in the studied lakes since the year 2000 (Fig. S3). Despite large among-lake differences in phytoplankton communities (Fig. 3D; Fig. S2A), division of the studied lakes in low and high TP (<30 µg l^{-1} and >30 µg l^{-1}, respectively) showed that eutrophication shifted the highest contributing taxa from diatoms (low TP: 24±2%, high TP: 13±2%) to cyanobacteria (low TP: 7±8%, high TP:34±3%). Nevertheless, cryptophytes, chrysophytes and raphidophytes were dominant taxa in three of the studied lakes (Fig. 3D; Fig. S2A). According to our size classification, the percentage of edible phytoplankton for zooplankton (<35 um) averaged 29.6±14.8% of all phytoplankton biomass across the studied lakes and was lowest in the highest TP lake (~4%; Fig. 3G). A negative linear trend was observed between edible biomass contribution and TP ($y=0.45 – 0.0024x$; $r^2=0.40$, $p=0.03$; Table S2). Changes in available biomass for zooplankton resulted from an increase in the share of filamentous and colony-forming algae to phytoplankton biomass with eutrophication. Correction of phytoplankton biomass and taxa community contribution based on size classification revealed that available biomass for zooplankton has much more variability across the TP gradient than whole phytoplankton biomass ($\ln(y)=2.46 + 0.75\ln(x)$; $r^2=0.55$, $p<0.01$; Fig. 3H). Of all taxa, cryptophytes and dinoflagellates dominated the edible phytoplankton biomass (35.5±19.5 and 21.2±19.9%, respectively; Fig. 3I). Zooplankton biomass increased 0.89% for every 1% increase in TP ($\ln(y)=1.12 + 0.89\ln(x)$; $r^2=0.60$, $p<0.01$; Fig. 3B; Table S2). The zooplankton community was stable across the TP gradient with copepods dominating the studied lakes (71±13%) and cyclopoids being the highest contributor to total copepod biomass (70±21%) (Fig. 3E). At the species level, eutrophication did not show clear trends across the studied lakes (Fig. S2B). Fish relative biomass (accounted as biomass per unit effort, BPUE) increased linearly with TP ($y= 839.2 + 20.4x$; $r^2= 0.72$, $p<0.001$; Fig. 3C; Table S2). Fish communities switched from percids to cyprinids with eutrophication, driven by a decrease in perch contribution with respect to roach, bream and white bream (Fig. 3F; Fig. S2C). Salmonids were only present in the most oligotrophic lakes while contributions from other fish species to fish communities varied with TP (Fig. S4).

3.2. Fatty acid volumetric production and productivity

Total volumetric production (TVP) relationship with TP was best described by a Beverton-Holt density-dependent model (RMSE=84.04, a: p=0.006, b: p=0.212; Fig. 4A; Table S3). Given the volumetric nature of TVP, we studied the relationship between TVP and phytoplankton biomass. This relationship was also best described by a Beverton-Holt density-dependent model (RMSE=59.21; a: p<0.01, b: p=0.03; Fig. 4C; Table S3) and presented a better fit than the TVP-TP model based on RMSE. This non-linear model indicates that TVP achieves half its asymptotic value at biomass ~850 µg C l^{-1}. Maximal asymptotic TVP was 377.9 pg FA l^{-1} Hr^{-1} (95% confidence intervals: 267.5–613.6 pg FA l^{-1} Hr^{-1}). Volumetric production of the high nutritional value FA EPA followed a logarithmic relationship with TP (y=0.23ln(x) - 0.094; RMSE=0.31, p=0.031; Fig. 4D; Table S3). Lake Lohjanjärvi (TP= 37 µg l^{-1}), which had the highest observed diatom biomass (1065 µg C l^{-1}), had >600% more EPA volumetric production than the rest of the studied lakes (Fig. 4D) while presenting an average 2xSecchi depth to chlorophyll-a value (0.1 compared to 0.6±1.0 of all lakes). Model fitting highlighted this lake as an outlier since its standardized residual in both linear and logarithmic models was ~3 (data not shown), while non-linear model fitting did not converge to a solution. Therefore, we excluded this lake when fitting the presented volumetric EPA production model (Fig. 4D). DHA volumetric production relationship with TP was best described by a Ricker model (RMSE=0.92; a: p=0.08, *Pp:* p=0.02; Fig. 4F; Table S2). Nevertheless, large variation in the values of DHA volumetric production across the TP gradient was observed, leading to significant uncertainty in the estimates of the models' coefficients. Total Productivity followed a Ricker density-dependent model (RMSE=12.85, a: p<0.001, Pp: p<0.001; Fig. 4B; Table S3). Based on our model, the TP concentration of maximal productivity was 32 µg l^{-1} (95% confidence interval: 28–39 µg l^{-1}; Fig. 4B). EPA and DHA productivity relationship with TP was best described by the same model than Total Productivity (EPA: RMSE=0.20; a: p=0.070, *Pp*: p=0.016; DHA: RMSE=0.14; a: p=0.052, *Pp:* p=0.017; Fig. 4E,G; Table S3). Despite one of the non-linear regression terms was slightly above significance for EPA, 95% confidence intervals of model coefficients did not include zero and frequency plots resembled a normal distribution (Table S3). For DHA productivity, frequency plots of bootstrapped model coefficients were skewed, indicating uncertainty in coefficient values and hence the null hypothesis cannot be rejected. Based on our results, the TP concentrations of maximal EPA and DHA productivity were 35 and 22 µg l^{-1}, respectively (95% confidence intervals: 22–61 and 17–32 µg l^{-1} for EPA and DHA respectively; Fig. 4E,G). Given the possible contribution of non-phytoplankton organic material to seston dry weight in low TP lakes, we assessed if volumetric production per phytoplankton biomass (VPPB) across our TP gradient equated our productivity results (Fig. S5). EPA and DHA VPPB relationship with TP was best described by a non-linear Ricker model (Fig. S5A,B; Table S3), while a log-log transformed model presented the best fit for total VPPB (Fig. S5C).

3.3. Changes in productivity and fatty acid content of food web components

Given the importance of EPA and DHA production for aquatic food webs, phytoplankton EPA and DHA productivity results were compared with the content of these FAs at higher trophic levels (Fig. 5). EPA contents were highest in cladocerans (13.5±5.3 mg g^{-1}), followed by copepods (6.8±3.8 mg g^{-1}) and fish (smelt 2.1±1.6, roach 1.0±0.9, perch 0.8±0.6, ruffe 0.7±0.5 mg g^{-1} respectively). DHA contents were highest in copepods (11.3±6.7 mg g^{-1}), followed by fish (smelt 3.7±2.9, perch 2.5±2.1, ruffe 1.8±1.4, roach

1.5±1.0 mg g^{-1} respectively) and cladocerans (0.8±0.5 mg g^{-1}). As previously mentioned, EPA and DHA productivity was highest between TP concentrations of 35 and 22 µg l^{-1}, respectively (Fig. 5A,H). Herbivorous cladocerans ($\ln(y)=14.67 - 0.071x$; $r^2=0.32$, $p=0.027$; Table S4) and calanoids ($\ln(y)=3.61 - 0.44\ln(x)$; $r^2=0.38$, $p=0.032$; Table S4) showed decreased EPA and DHA contents (respectively) with increases in TP. Predatory cladocerans EPA content did not present a significant effect of TP when using the model with lowest observed AIC (Fig. 5C). Cyclopoids DHA content response to TP increases was best described by a Ricker model. The TP concentration of maximal cyclopods DHA content was 34 µg l^{-1} (95% confidence interval: 26–47 µg l^{-1}; Fig. 5J). EPA content of calanoids and cyclopoids did not vary with eutrophication (Fig S6). Smelt EPA and DHA content relationship with TP followed a Ricker model (EPA: RMSE=1.5; a: $p<0.01$, Pp: $p<0.01$; DHA: RMSE=2.1; a: $p<0.01$, *Pp:* $p<0.01$; Fig. 5D,K; Table S4). Maximal smelt EPA and DHA contents were observed at TP 33 and 29 µg l^{-1}, respectively (95% confidence intervals: 27–44 and 25–35 for EPA and DHA respectively; Fig 4D,K). Different responses to eutrophication were found in perch and ruffe (Fig. 5E,G;M,O): perch decreased both EPA ($\ln(y)=-0.08 - 0.15\ln(x)$; $r^2=0.02$, $p=0.04$; Table S4) and DHA ($y=1.48 - 0.29x$; $r^2=0.09$, $p<0.01$; Table S4) content with increases in TP while ruffe did not present a significant effect (Fig. 5E,G;M,O). Roach, one of the cyprinid fish species whose contribution to community composition was favoured by eutrophication, showed no effect of eutrophication on EPA or DHA content (Fig. 5F,N).

4. Discussion

Eutrophication significantly affected food web biomass and structure, community composition, and phytoplankton production and productivity, but its effects on the nutritional value of consumers were variable. Total food web biomass increased along the TP gradient, but the rate of increase was not equal among the studied trophic levels. Between phytoplankton and zooplankton, phytoplankton biomass increased more per increase in TP than zooplankton. Proliferation of phytoplankton with eutrophication has long been recognized in different lakes (Vollenweider et al., 1974; Hanson & Leggett, 1982; Carpenter et al., 2001). In addition, previous work on whole food webs found similar alterations of food web structure with eutrophication as in this study, highlighting that phytoplankton and fish respond more strongly than zooplankton to increases in nutrients (e.g. Jeppesen et al., 2000; Carpenter et al., 2001; Keva et al., 2021). Overall, our results agree with the 'Green World Hypothesis', where primary producers (phytoplankton) are controlled bottom-up by nutrients and primary consumers (zooplankton) are controlled top-down by fish (Hairston et al., 1960).

Phytoplankton community composition plays a critical role in the availability of micronutrients such as PUFAs and sterols for higher trophic levels (Brett & Müller-Navarra, 1997; Müller-Navarra et al., 2000, von Elert et al., 2003; Taipale et al., 2016a). Moreover, changes in community composition can also alter the edibility of phytoplankton biomass, since herbivorous zooplankton preferentially graze on small (<35 µm) phytoplankton cells (Porter, 1973; Knisely & Geller, 1986; Heathcote et al., 2016; Lürling, 2021). Our results suggest that eutrophication not only shifts phytoplankton communities from diatom to cyanobacteria dominated, but also decreases the contribution of preferentially grazed (<35µm) phytoplankton. It is possible that such changes are a result of targeted zooplankton predation on smaller sized and nutritious phytoplankton (Meise et al., 1985; Meunier et al., 2016; Lürling, 2021), resulting in dominance of low-quality phytoplankton in the water column with increasing TP. Interestingly, edible

phytoplankton communities were stable with eutrophication, highlighting that these taxa could play important roles for zooplankton nutrition. However, our phytoplankton biomass estimates did not include picophytoplankton due to the difficulty of identifying such cells with Lugol-preserved samples. Year-round seasonality of phytoplankton communities also has to be considered since the presented data represents a single point in time. Fish communities shifted from percid to cyprinid-dominated due to a decrease in perch and an increase in roach, bream and white bream. This shift was accompanied with a pronounced increase in fish relative biomass. These results agree with previous research showing that eutrophication favours cyprinid over percid and salmonid populations and increases overall fish biomass in lakes (Persson, 1991; Olin et al., 2002; Hayden et al., 2017).

To our knowledge, this is the first work where CSIA was used to study how production of FAs is affected by lake nutrient status. Volumetric production and productivity, the units proposed in this work, represent a snapshot in time of how inorganic carbon is metabolically channelled to FAs in phytoplankton. TVP and total productivity could be used as proxies of primary production, while individual FA production can give insight on the rate at which these molecules are synthesised and are available for higher trophic levels. As we hypothesised, TVP did not increase linearly with TP and a non-linear model best described the data. Nevertheless, the variability in TVP observed at high TP increased the uncertainty in model coefficients. The relationship between TVP and phytoplankton biomass also followed a non-linear model (Beverton-Holt), suggesting lower production efficiency at high phytoplankton biomass (>850 µg C l^{-1}). Based on our results and previous literature (Schindler, 1977), phytoplankton biomass linearly increases with TP, nevertheless, our results challenge the idea that volumetric production follows the same trend by proposing the existence of a limit (asymptote) to how much production phytoplankton biomass can achieve. EPA volumetric production responded logarithmically to increases in TP while the model that best described DHA volumetric production relationship with TP was a non-linear Ricker model. Despite that volumetric production of these PUFA presents the same constrains than TVP (variation of phytoplankton biomass with TP), more extreme outliers were observed. These extreme values can be explained by large differences in phytoplankton communities and biomass, since not all taxa can produce EPA and DHA (Ahlgren et al., 1992; Lang et al., 2011, Taipale et al., 2013). For example, the highest EPA volumetric production observation in our dataset was from a diatom dominated eutrophic lake (TP= 37 µg l^{-1}), where diatom biomass was the highest of all studied lakes. Interestingly, the highest DHA volumetric production occurred in a cryptophyte dominated eutrophic lake (TP= 76 µg l^{-1}). Changes in total productivity across the TP gradient shed light on how fixation of inorganic carbon per phytoplankton biomass is affected by eutrophication. The unimodal relationship observed between total productivity−TP suggests that peak productivity occurs at a TP concentration of 32 µg l^{-1} after which productivity is reduced. These results agree with the "Paradox of enrichment", where at high nutrient availability, further increases in nutrients reduce productivity (Rosenzweig, 1971). The relationship between both EPA and DHA productivity and TP was best described by the same non-linear model (Ricker model) than total productivity. The observed unimodal response of EPA and DHA productivity to increases in nutrients agrees with previous work on estimation of phytoplankton EPA and DHA levels at different lake trophic status based on phytoplankton communities (Taipale et al., 2016b). Overall, the estimated TP concentrations of maximal total, EPA, and DHA productivities suggest that lakes within a TP range of 22−35 µg l^{-1} possess the highest FA production and PUFA quality per

phytoplankton biomass. We hypothesize that at TP concentrations >35 µg l^{-1}, higher cell densities increase competition for light between phytoplankton cells overall decreasing photosynthetic output per cell (Han et al., 2000; Day et al., 2012). It is also possible that the dominant phytoplankton communities at high TP are on average less photosynthetically efficient than communities at lower TP or that they differ in the rate of FA production (Kong et al., 2021; Uwizeye et al., 2021). In oligotrophic lakes on the other hand, phytoplankton production is likely limited by nutrients on a per cell basis.

Analysis of EPA and DHA content of different food web components allow the study of how changes in phytoplankton production of these PUFAs affect higher trophic levels. We observed that EPA and DHA contents do not follow the expected trends in the studied food web components and that some organisms are more affected than others by the effects of eutrophication. Within zooplankton, herbivorous cladocerans and calanoids decreased their EPA and DHA content (respectively) with increases in TP, while predatory cladocerans EPA content did not show a significant effect of TP. Cyclopoids DHA content presented the same non-linear (Ricker model) response to TP than DHA productivity, with peak DHA been observed at higher TP that phytoplankton maximal DHA productivity (34 compared to 22 µg l^{-1}, respectively). Despite low EPA and DHA productivity at low TP (<20 µg l^{-1}), herbivorous cladocerans EPA and calanoids DHA contents were highest while increases in TP lead to decreases in the content of these PUFAs. We hypothesise that this mismatch between productivity and cladocerans and calanoids content could be a result of edible phytoplankton contributing to only a portion of productivity. Since small phytoplankton cells are preferentially grazed by zooplankton (Porter, 1973; Knisely & Geller, 1986; Heathcote et al., 2016; Lürling, 2021), all EPA and DHA produced by large phytoplankton (>35 um) cannot be efficiently transferred to zooplankton. This effect is perhaps more visible in our highest EPA productivity lake (Lohjanjärvi, TP= 37) where the high diatom biomass is likely contributing a large share of the EPA produced (Taipale et al., 2016b). Despite this high EPA productivity, most diatom biomass is not available for zooplankton due to its size (>35 um). Unfortunately, given the variability in FA content between phytoplankton species, and the effects of physicochemical parameters on FA profiles (Lang et al., 2011; Kong et al., 2021), it is not possible to accurately estimate how much of the EPA or DHA production comes from edible phytoplankton (<35 um). New insights into EPA and DHA biosynthesis and bioconversion in copepods (Boyen et al., 2023) suggest that different zooplankton species could have adaptations to varying availability of EPA and DHA in phytoplankton, making estimations of FA transfer across trophic levels more complex. In addition, differences in life-cycle length and lipid storage capabilities (Hiltunen et al., 2016), together with differential channelling of FAs to somatic growth, reproduction or catabolism and respiration (Galloway & Budge, 2020) could contribute to differences between predated phytoplankton and FA content in zooplankton.

Of the studied fish species, smelt (a short lived pelagic planktivorous fish) directly followed the phytoplankton EPA and DHA productivity curves and presented peak contents at 33 and 29 µg l^{-1} TP for EPA and DHA, respectively. A decrease in EPA and DHA contents of Perch (generalist) was observed with eutrophication. The fact that EPA and DHA contents of ruffe (benthivorous), the other studied percid species, was not affected by eutrophication suggest that large physiological differences in fatty acid metabolism between fish species exist. Perch is known to have the genetic ability to elongate DHA from shorter chain length PUFAs, hence it is possible that other percids (and non-percids) have the same ability (Geay et al., 2016; Kabeya et al., 2018; Ishikawa

et al., 2019). We do not have data on littoral and profundal benthos, which could partially explain the results observed. Keva et al. (2021) did not observe a correlation between the EPA and DHA contents of littoral benthos but found a negative trend with profundal invertebrates along a TP and temperature gradient. Benthic invertebrates have very low amounts of EPA and DHA compared to zooplankton irrespective of lake type (Vesterinen et al., 2021). Nevertheless, we highlight that food web components not sampled in this work (such as periphyton, benthic and pelagic macroinvertebrates, and others) may contribute to varying extents in the supply of energy and micronutrients to higher trophic levels and should be accounted for in whole food webs analysis. EPA and DHA contents of fish results partially agree with previous studies where decreases in seston and zooplankton nutritional quality in eutrophic lakes were efficiently mitigated by freshwater fish species such as ruffe and roach (Kainz et al., 2017; Keva et al., 2021; Gomes et al., 2021; Taipale et at., 2022a). The lack of consistency in EPA and DHA contents across different food web components underlines the complexity of the studied systems and suggest that variables such as species composition, PUFA synthesis and elongation capacity, differences in FA metabolism, life-cycle length and lipid storage capabilities play an increasingly balancing role towards higher trophic levels. This is especially important to consider when environmental changes produce a strong effect on phytoplankton and the repercussion of such changes are extrapolated to higher trophic levels.

5. Conclusions

This study showed that eutrophication increases the biomass of phytoplankton, zooplankton, and fish in boreal lakes while altering the community composition of these trophic levels. Additionally, eutrophication reduced the contribution of phytoplankton that is preferentially grazed by zooplankton (<35µm). Analysis of phytoplankton fatty acid production showed that there is an optimal nutrient level (range 22−35 µg l^{-1} TP) where production per biomass is maximized. The unimodal production of phytoplankton EPA and DHA indicates lower availability of these essential micronutrients in oligotrophic and highly eutrophic lakes. Of the studied food web components, cyclopoid DHA and smelt (pelagic planktivorous fish) EPA and DHA contents replicated the trends observed in phytoplankton production. However, in other species this pattern was not observed, suggesting that other mechanisms such as selective grazing, bioconversion of PUFAs, lipid storage and differential channelling of FAs to different metabolic processes play a role in shaping the EPA and DHA content of some consumers with eutrophication. Future work will show if the observed nutrient range of maximal production holds in a wider set of lakes from different regions, and if this higher production transfers to higher trophic levels.

CRediT authorship contribution

M.L.C., P.S., E.P. and S.J.T. planned and designed the research. All authors were involved in data acquisition. M.L.C. analysed the data. M.L.C., K.K.K., P.S., E.P., and S.J.T. interpreted the data. M.L.C. wrote the manuscript in collaboration with K.K.K, P.S., E.P., and S.J.T. All authors commented on the manuscript.

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. At all stages of this study, national guidelines (FI-564/2013 & FI-487/2013) and the European

Union directive (2010/63/EU) on the protection of animals used for scientific purposes were applied.

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Data availability statement

All data as exportable files and data analysis scripts necessary to replicate the results presented in this study are available at (link will be provided upon publication). A comprehensive description of the content of each file, and the description of each Supporting dataset (Dataset S) can be found in the same DOI under the name "Metadata_file."

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Figure legends

Graphical Abstract

Fig. 1.

Map of study region and sampled lakes in central and southern Finland.

Fig. 2.

Diagram of sampling methods used in this study. For a detailed description please see the Materials and Methods section.

Fig. 3.

Changes in community biomass, relative biomass, and composition across a total phosphorus (TP) gradient (4–140 μg l^{-1}). Total and edible phytoplankton biomass (A and H, respectively) and community composition (D and I, respectively) were derived from taxa morphology-specific geometric formulas of observed phytoplankton with microscopy. Phytoplankton were classified as edible based on size (<35μm, see materials and methods: Community composition and biomass) and their proportional change with TP is presented in (G). Zooplankton biomass (B) and community composition (E) were derived from species-specific carbon regressions. Fish relative biomass (C) and community composition (F) were obtained using calculated biomass per unit of effort (BPUE) for each lake. Regression models (A, B, C, G, and H) display Root Mean Square Error (RMSE), p-value (p) and coefficient of determination (r^2). For all models, information is available in Table S2.

Fig. 4.

Non-linear models of changes in total, EPA, and DHA volumetric production (A, D, and F, respectively) and productivity (B, E, and G, respectively) across a total phosphorus (TP) gradient (4–140 μg l^{-1}) and changes in total volumetric production across increases in phytoplankton biomass (C). Volumetric production refers to the fatty acid (FA) production per litre per hour (pg FA l^{-1} Hr^{-1}), while productivity refers to FA production per mg of seston dry weight per hour (ng FA l^{-1} Hr^{-1}). Density dependent non-linear models of the form Beverton-Holt (A and C) and Ricker (B, E, F, and G) present p-values obtained through parametric tests for each parameter (Beverton-Holt: a, b; Ricker: a, Pp; see materials and methods: Data analysis). Logarithmic model (D) display coefficient of determination (r^2) and p-value (p) of term log(x). Dashed lines represent non-significant models ($p>0.05$). All models display Root Mean Square Error (RMSE). For all models, information is available in Table S3.

Fig. 5.

Changes in EPA and DHA productivity by phytoplankton (A and H, respectively) and regressions models of EPA content in herbivorous (B) and predatory Cladocera (C), DHA content in Calanoida (I) and Cyclopoida (J), as well as EPA and DHA content of fish species studied, smelt (D and K, respectively), ruffe (E and M, respectively), roach (F and

N, respectively) and perch (G and O, respectively) across a total phosphorus (TP) gradient (4–140 µg l^{-1}). Productivity refers to fatty acid (FA) production per mg of seston dry weight per hour (ng FA l^{-1} Hr^{-1}). Density dependent non-linear models of the form Ricker (A, D, H, J, and K) present p-values obtained through parametric tests for each parameter (Ricker: a, Pp; see materials and methods: Data analysis). Linear and logarithmic regression models display p-value (p) and coefficient of determination (r^2). Dashed lines represent nonsignificant coefficient p-values ($p>0.05$). All models display Root Mean Square Error (RMSE). For all models, information is available in Table S4.

Table legends

Table 1 Chemical and physical characteristics of the study lakes. Lat (°N), Long (°E) refers to latitude and longitude, Chl-a = chlorophyll-a, TN = total nitrogen, DIN = dissolved inorganic nitrogen, TP = total phosphorus, DOC = dissolved organic carbon, DIC = dissolved inorganic carbon, Surface temperature (°C) represents the average of temperature measurements conducted every 1 m from the surface to the 2xSecci depth.

Figures

Graphical Abstract

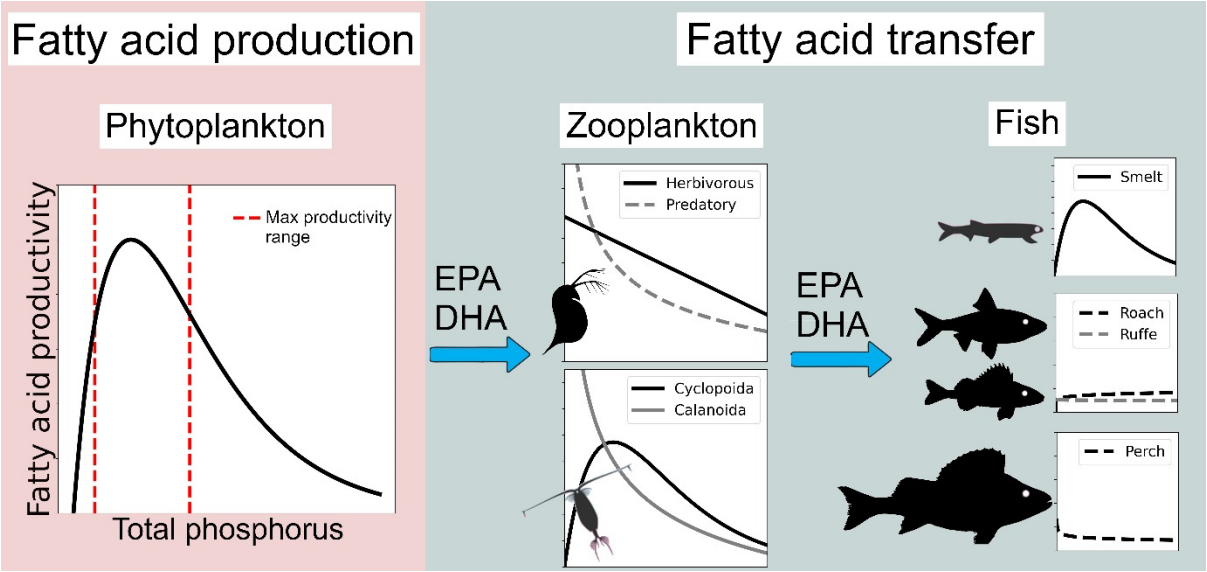


Fig. 1.

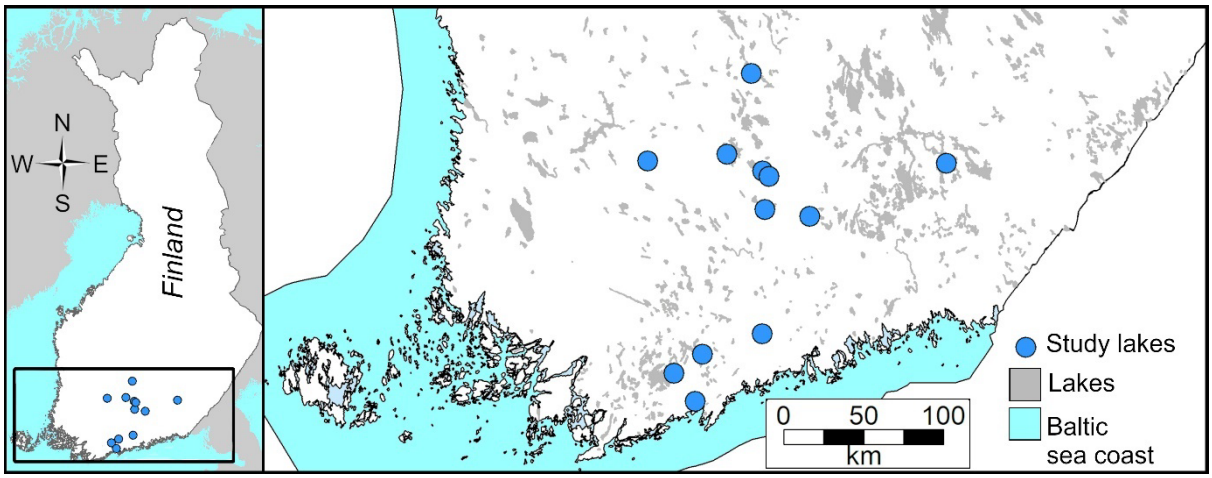


Fig. 2.

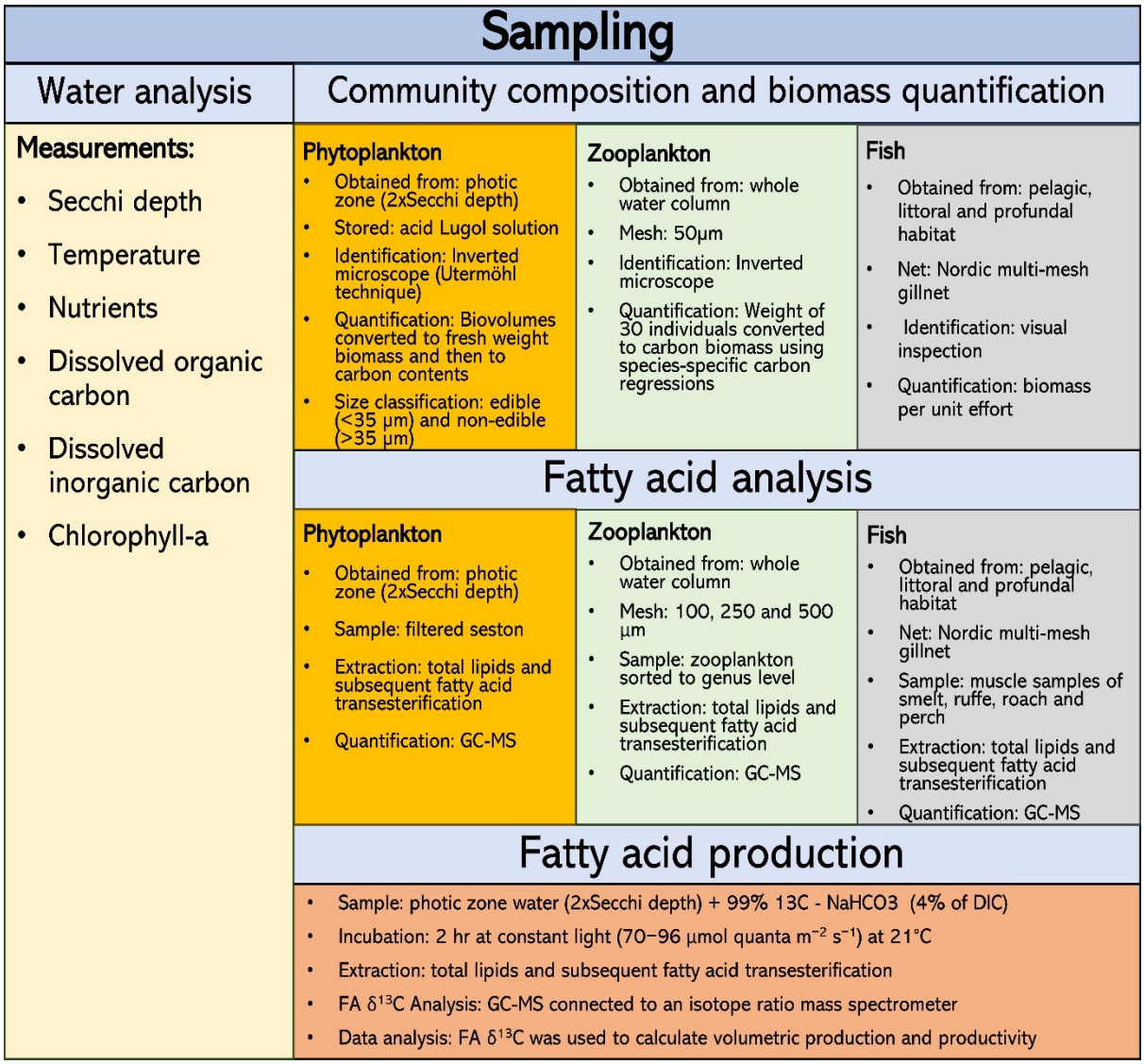

Sampling
Water analysis
Community composition and biomass quantification
Measurements:
• Secchi depth
• Temperature
• Nutrients
• Dissolved organic carbon
• Dissolved inorganic carbon
• Chlorophyll-a
Phytoplankton
• Obtained from: photic zone (2xSecchi depth)
• Stored: acid Lugol solution
• Identification: Inverted microscope (Utermöhl technique)
• Quantification: Biovolumes converted to fresh weight biomass and then to carbon contents
• Size classification: edible (<35 µm) and non-edible (>35 µm)
Zooplankton
• Obtained from: whole water column
• Mesh: 50µm
• Identification: Inverted microscope
• Quantification: Weight of 30 individuals converted to carbon biomass using species-specific carbon regressions
Fish
• Obtained from: pelagic, littoral and profundal habitat
• Net: Nordic multi-mesh gillnet
• Identification: visual inspection
• Quantification: biomass per unit effort
Fatty acid analysis
Phytoplankton
• Obtained from: photic zone (2xSecchi depth)
• Sample: filtered seston
• Extraction: total lipids and subsequent fatty acid transesterification
• Quantification: GC-MS
Zooplankton
• Obtained from: whole water column
• Mesh: 100, 250 and 500 µm
• Sample: zooplankton sorted to genus level
• Extraction: total lipids and subsequent fatty acid transesterification
• Quantification: GC-MS
Fish
• Obtained from: pelagic, littoral and profundal habitat
• Net: Nordic multi-mesh gillnet
• Sample: muscle samples of smelt, ruffe, roach and perch
• Extraction: total lipids and subsequent fatty acid transesterification
• Quantification: GC-MS
Fatty acid production
• Sample: photic zone water (2xSecchi depth) + 99% 13C - NaHCO3 (4% of DIC)
• Incubation: 2 hr at constant light (70–96 µmol quanta m^{-2} s^{-1}) at 21°C
• Extraction: total lipids and subsequent fatty acid transesterification
• FA $\delta^{13}C$ Analysis: GC-MS connected to an isotope ratio mass spectrometer
• Data analysis: FA $\delta^{13}C$ was used to calculate volumetric production and productivity

Fig. 3.

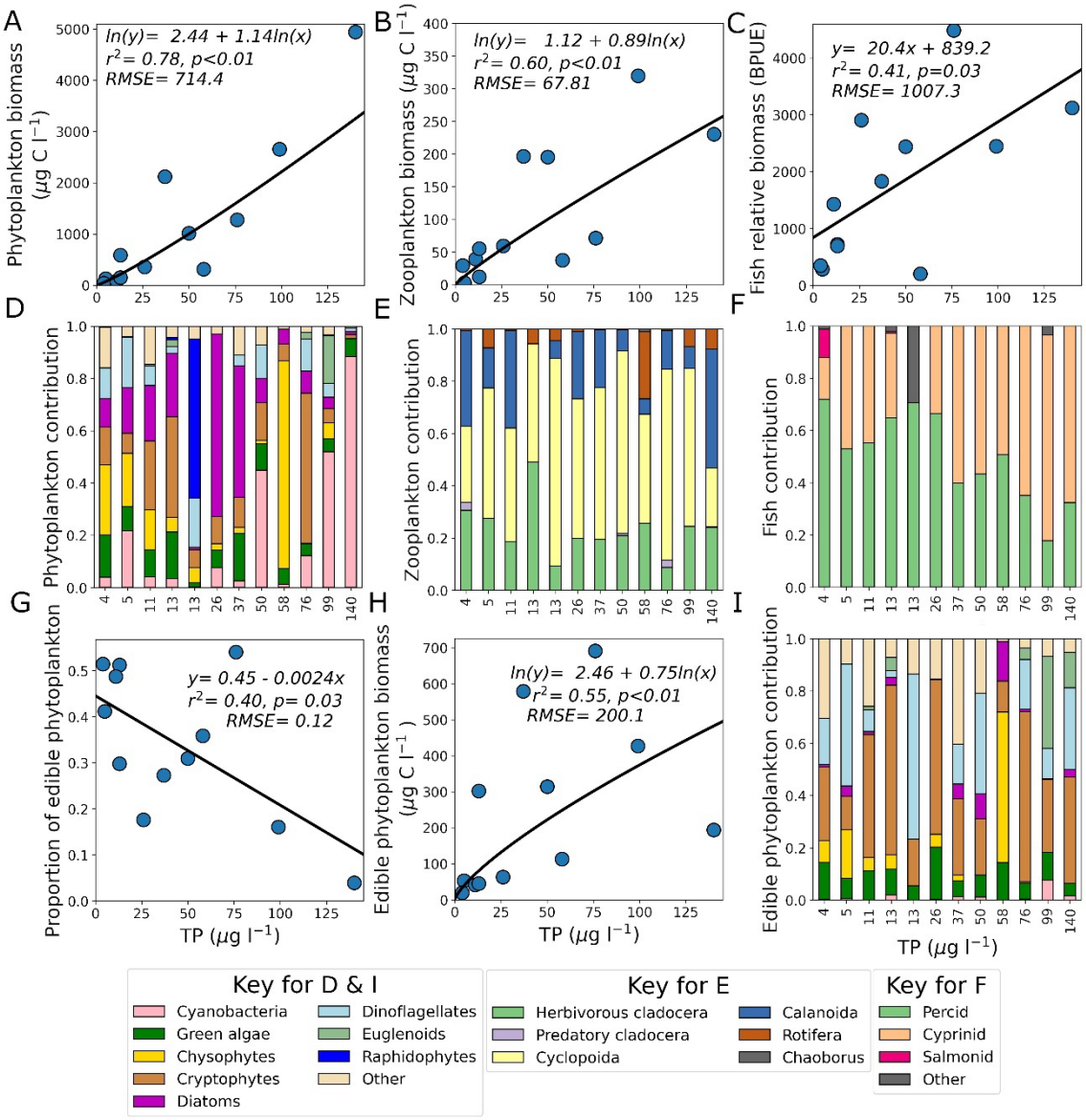

A
ln(y)= 2.44 + 1.14ln(x)
r²= 0.78, p<0.01
RMSE= 714.4
Phytoplankton biomass (μg C l⁻¹)
B
ln(y)= 1.12 + 0.89ln(x)
r²= 0.60, p<0.01
RMSE= 67.81
Zooplankton biomass (μg C l⁻¹)
C
y= 20.4x + 839.2
r²= 0.41, p=0.03
RMSE= 1007.3
Fish relative biomass (BPUE)
D
Phytoplankton contribution
E
Zooplankton contribution
F
Fish contribution
G
y= 0.45 - 0.0024x
r²= 0.40, p= 0.03
RMSE= 0.12
Proportion of edible phytoplankton
TP (μg l⁻¹)
H
ln(y)= 2.46 + 0.75ln(x)
r²= 0.55, p<0.01
RMSE= 200.1
Edible phytoplankton biomass (μg C l⁻¹)
TP (μg l⁻¹)
I
Edible phytoplankton contribution
TP (μg l⁻¹)
Key for D & I
Cyanobacteria
Green algae
Chysophytes
Cryptophytes
Diatoms
Dinoflagellates
Euglenoids
Raphidophytes
Other
Key for E
Herbivorous cladocera
Predatory cladocera
Cyclopoida
Calanoida
Rotifera
Chaoborus
Key for F
Percid
Cyprinid
Salmonid
Other

Fig. 4.

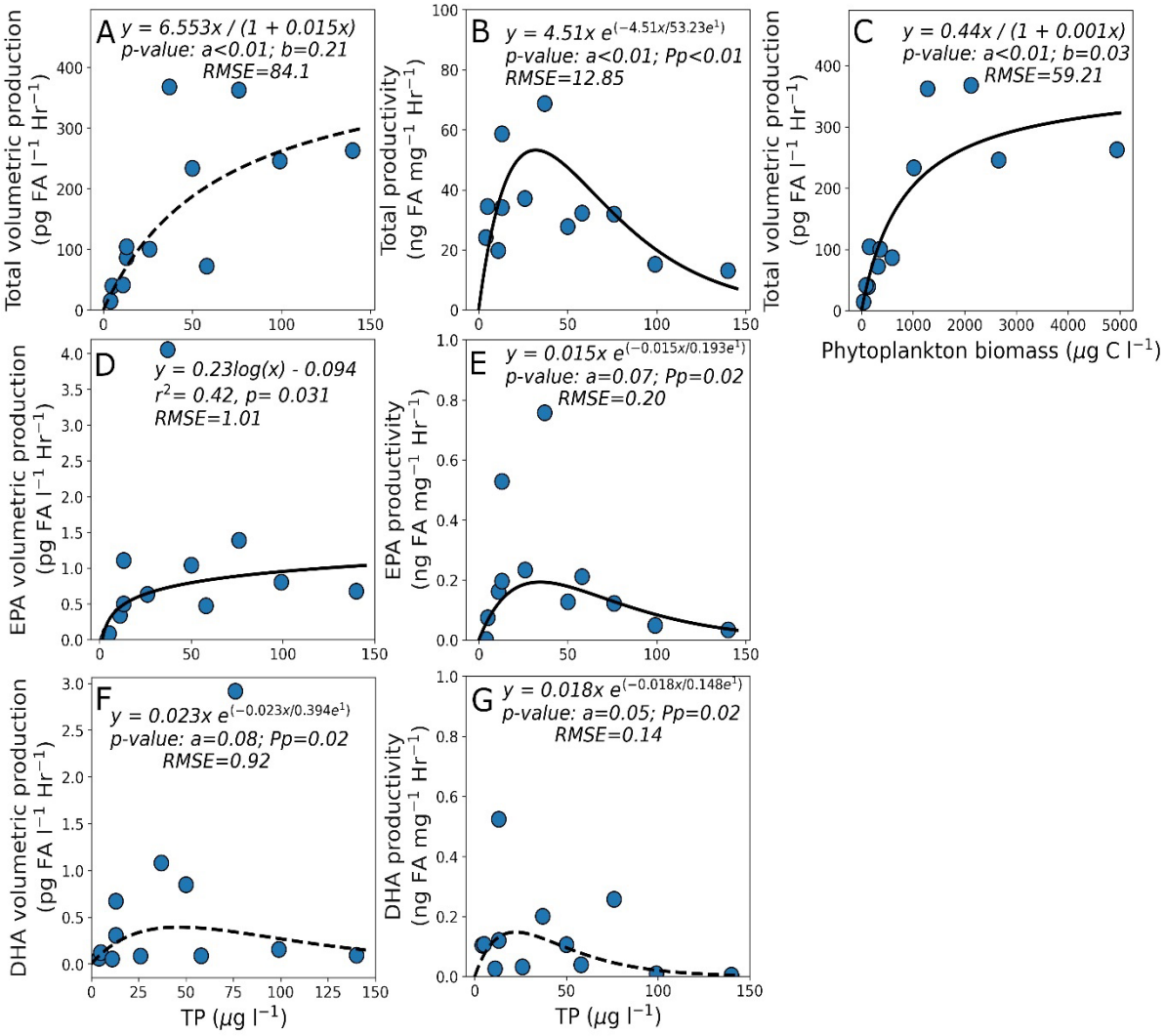
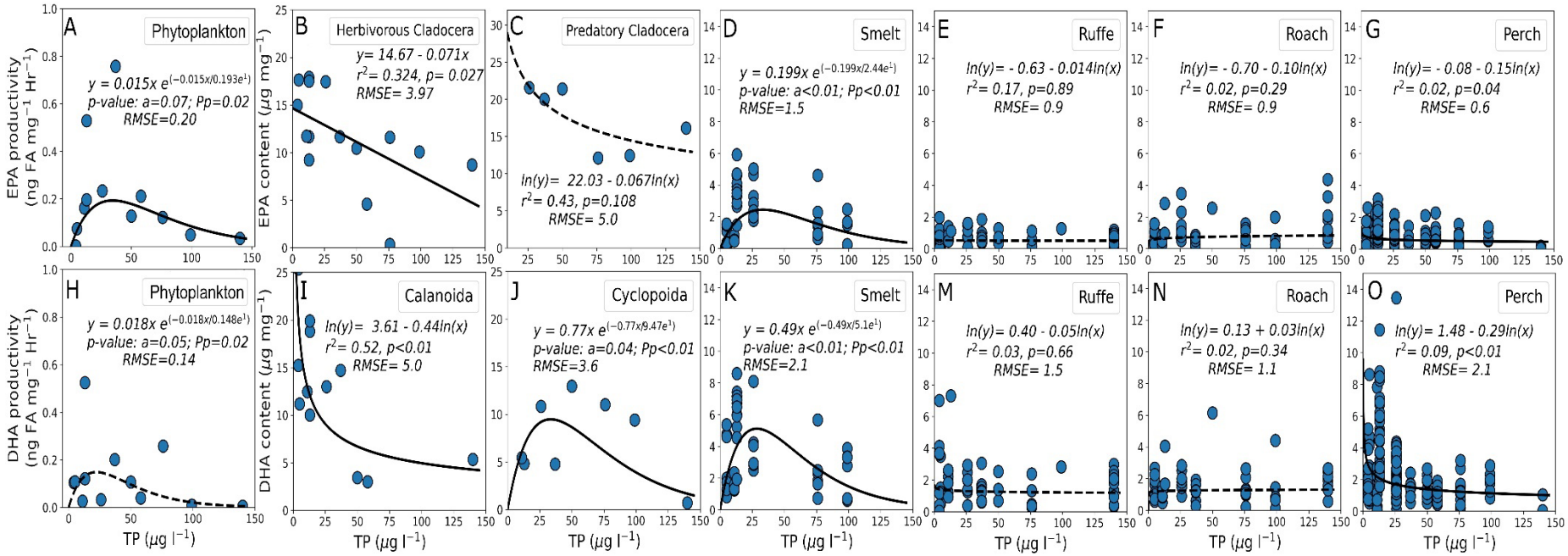

A
y = 6.553x / (1 + 0.015x)
p-value: a<0.01; b=0.21
RMSE=84.1
Total volumetric production (pg FA l−1 Hr−1)
B
y = 4.51x e(−4.51x/53.23e1)
p-value: a<0.01; Pp<0.01
RMSE=12.85
Total productivity (ng FA mg−1 Hr−1)
C
y = 0.44x / (1 + 0.001x)
p-value: a<0.01; b=0.03
RMSE=59.21
Total volumetric production (pg FA l−1 Hr−1)
Phytoplankton biomass (µg C l−1)
D
y = 0.23log(x) - 0.094
r2= 0.42, p= 0.031
RMSE=1.01
EPA volumetric production (pg FA l−1 Hr−1)
E
y = 0.015x e(−0.015x/0.193e1)
p-value: a=0.07; Pp=0.02
RMSE=0.20
EPA productivity (ng FA mg−1 Hr−1)
F
y = 0.023x e(−0.023x/0.394e1)
p-value: a=0.08; Pp=0.02
RMSE=0.92
DHA volumetric production (pg FA l−1 Hr−1)
TP (µg l−1)
G
y = 0.018x e(−0.018x/0.148e1)
p-value: a=0.05; Pp=0.02
RMSE=0.14
DHA productivity (ng FA mg−1 Hr−1)
TP (µg l−1)

Fig. 5.


A
Phytoplankton
y = 0.015x e(−0.015x/0.193e1)
p-value: a=0.07; Pp=0.02
RMSE=0.20
B
Herbivorous Cladocera
y= 14.67 - 0.071x
r2= 0.324, p= 0.027
RMSE= 3.97
C
Predatory Cladocera
ln(y)= 22.03 - 0.067ln(x)
r2= 0.43, p=0.108
RMSE= 5.0
D
Smelt
y = 0.199x e(−0.199x/2.44e1)
p-value: a<0.01; Pp<0.01
RMSE=1.5
E
Ruffe
ln(y)= - 0.63 - 0.014ln(x)
r2= 0.17, p=0.89
RMSE= 0.9
F
Roach
ln(y)= - 0.70 - 0.10ln(x)
r2= 0.02, p=0.29
RMSE= 0.9
G
Perch
ln(y)= - 0.08 - 0.15ln(x)
r2= 0.02, p=0.04
RMSE= 0.6
EPA productivity (ng FA mg−1 Hr−1)
EPA content (µg mg−1)
H
Phytoplankton
y = 0.018x e(−0.018x/0.148e1)
p-value: a=0.05; Pp=0.02
RMSE=0.14
I
Calanoida
ln(y)= 3.61 - 0.44ln(x)
r2= 0.52, p<0.01
RMSE= 5.0
J
Cyclopoida
y = 0.77x e(−0.77x/9.47e1)
p-value: a=0.04; Pp<0.01
RMSE=3.6
K
Smelt
y = 0.49x e(−0.49x/5.1e1)
p-value: a<0.01; Pp<0.01
RMSE=2.1
M
Ruffe
ln(y)= 0.40 - 0.05ln(x)
r2= 0.03, p=0.66
RMSE= 1.5
N
Roach
ln(y)= 0.13 + 0.03ln(x)
r2= 0.02, p=0.34
RMSE= 1.1
O
Perch
ln(y)= 1.48 - 0.29ln(x)
r2= 0.09, p<0.01
RMSE= 2.1
DHA productivity (ng FA mg−1 Hr−1)
DHA content (µg mg−1)
TP (µg l−1)

Tables

Table 1

Lake	Lat, Long (°N;°E)	Lake area (km^2)	Mean depth (m)	Max depth (m)	Chl-a ($\mu g\ l^{-1}$)	P-PO4 ($\mu g\ l^{-1}$)	N-NH4 ($\mu g\ l^{-1}$)	NO2+ NO3 ($\mu g\ l^{-1}$)	TN ($\mu g\ l^{-1}$)	DIN: TP	TN:TP	TP ($\mu g\ l^{-1}$)	DOC ($\mu g\ C\ l^{-1}$)	Phyto-plankton biomass ($\mu g\ C\ l^{-1}$)	DIC (mg $C\ l^{-1}$)	2xSecchi depth (m)	Surface temperature (°C)	Sampling date
Kukkia	61.32', 24.64'	47	5.2	35.6	5.1	2	7	15	509	2	46.3	11	6.9	85.1	0.66	4.5	20.8	29/07/2021
Isojärvi	61.72', 24.92'	18.3	16.4	69.7	6.6	2	8	46	415	10.8	83	5	10.8	128.7	0.33	5	20.7	19/07/2021
Korpijärvi	61.28', 27.18'	31.2	10	41	2.3	2	14	55	413	17.25	103.3	4	6	37.6	0.49	9	18.6	27/07/2021
Tuusulan-järvi	60.44', 25.05'	6	3.2	10	35	5	6	13	965	0.25	12.7	76	7.8	1278.4	2.03	1.5	25.8	13/07/2021
Hulaus-järvi	61.29', 23.73'	2.2	1.1	3.3	81	3	11	14	1318	0.25	13.3	99	12	2655.2	1.25	1.5	26	14/07/2021
Lohjan-järvi	60.24', 24.03'	122	12.7	54.5	22	2	15	78	791	2.51	21.4	37	9.3	2119.4	1.39	2	22.8	21/07/2021
Vesijärvi	61.01', 25.60'	108	6	40	8.2	3	5	9	542	0.54	20.8	26	5.9	357.5	1.67	5	21.4	28/07/2021
Valkea-Kotinen	61.24', 25.06'	0.04	3	7	8.2	2	11	5	584	1.23	44.9	13	12.7	152.5	0.4	2	21.7	15/07/2021
Majajärvi	61.21', 25.13'	0.03	4.6	12	26	11	12	8	1024	0.34	17.7	58	19	315.7	0.58	0.8	17.6	22/07/2021
Pääjärvi	61.05'; 25.08'	13.4	14.4	85	11	2	15	1076	1573	83.92	121	13	12.3	589.6	0.82	3	24.5	12/07/2021
Enäjärvi	60.34', 24.36'	5	3.5	10	131	3	6	19	1814	0.18	13	140	6.4	4943.2	1.73	0.8	23.1	20/07/2021
Vikträsk	60.11', 24.28'	1.87	4.49	15	40	2	6	7	644	0.26	12.9	50	10.8	1014.2	1.87	1.4	22.1	26/07/2021