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Physiological adaptation of the diatom *Pseudo-nitzschia delicatissima* under copper starvation

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Abstract :

In the open ocean and particularly in iron (Fe)-limited environment, copper (Cu) deficiency might limit the growth of phytoplankton species. Cu is an essential trace metal used in electron-transfer reactions, such as respiration and photosynthesis, when bound to specific enzymes. Some phytoplankton species, such as the diatom Pseudo-nitzschia spp. can cope with Cu starvation through adaptative strategies. In this study, we investigated the physiological strategies of the marine diatom P. delicatissima against Cu starvation. Compared to the control, Cu starvation inhibited growth by 35%, but did not induce any excess mortality. Despite the bottleneck measured in the electron flow of the photosynthetic chain, cells of P. delicatissima conserved their photosynthesis ability. This photosynthesis maintenance was accompanied by structural changes of membranes, where pigments and lipid composition were strongly modified. Diatoms also strongly modified their metabolism, by redirecting their C allocation to energy storage under the form of triglycerides. By maintaining essential metabolic functions and storing energy under the form of lipids, these physiological adaptations might be a strategy enabling this diatom to later bloom under the return of favorable nutritional condition.

Highlights

► The diatom *Pseudo-nitzschia delicatissima* was cultivated under Cu starvation. ► Cu starvation did not increase mortality but inhibited the diatom growth. ► Structural changes in membranes enabled diatoms to maintain photosynthesis. ► Modifications in pigments, lipid classes and fatty acids were quantified. ► C allocation was redirected to energy storage under the form of lipids.

Keywords : Bacillariophyta, Copper starvation, Physiology, Membranes, Lipid metabolism, Energy storage, Photosynthesis, Photosystem II

Introduction

Copper (Cu) is an essential redox-active element with two oxidation states Cu+ and Cu2+ that is essential for phytoplankton growth. When this element is bound to enzymes (e.g. in plastocyanin (Pcn) and cytochrome oxidase) it can be used in electron-transfer reactions from various metabolic functions such as respiration, and photosynthesis [1]. Two studies in oceanic waters reported an increase in phytoplankton biomass following Cu addition [2,3]. Coale [2] hypothesized that this increase was due to a decrease in microzooplankton grazing due to Cu toxicity, but Peers et al. [3] reported no impact of Cu addition on the zooplankton grazing rate. Two alternative hypotheses were postulated to explain the Cu-induced increase in phytoplankton growth in the subarctic Pacific Ocean. First, the phytoplanktonic Cu requirements of phytoplankton cells in these waters could be linked to their Fe status [3,4]. Fe limitation, which occurs in 50% of the global ocean, is pervasive in this oceanic region [5,6]. Fe-limited phytoplankton may need Cu for its role in a multicopper oxidase, that is part of an inducible Fe transport system [7,8]. Due to this enzyme, the Cu demand of certain species under Fe limitation is therefore largely increased [3,9,10], and addition of Cu could thus stimulate Fe acquisition and, indirectly, phytoplankton growth. For some diatoms, however, the Cu demand of the high-affinity Fe transport system may be low relative to other cellular Cu pools, such as Cu-containing plastocyanin and superoxide dismutase, and cytochrome c oxidase [4,9]. In particular, some oceanic species that needed to adapt to Fe limitation constitutively replaced cytochrome c6, which carries electrons between the cytochrome b6f and the photosystem I (PSI), by its Cu-containing analog plastocyanin (PCn [4]). In these cases, Cu could stimulate phytoplankton growth independently of Fe [3] especially at surface where Cu concentrations can be limiting due to consumption linked with the biological activity and to the presence of strong organic ligands [11–13]. Experimental studies

confirmed that diatom growth was stimulated with the addition of Cu as compared to Cu organic levels, even without Fe limitation [3,9,12].

Cu limitation can therefore induce deleterious effects on microalgal cells, such as inhibiting growth rate [9,10], photosynthesis, carbon fixation, respiration [4,14,15]. While some strains can mitigate Cu limitation stress, the research on the physiological adaptations of microalgae to Cu limitation remains scarce. Photophysiology was especially studied as compared to other metabolic pathways. The main physiological response to Cu deficiency is the switch between the Cu-containing Pcn to the Fe-containing cytochrome c6 [4,14,16] to mitigate the bottleneck in the electron transfer. Modifications in the electron carrier can be accompanied by larger modifications of the photosynthetic chains. Some strains of the diatom *Thalassiosira oceanica* can modify their light harvesting complexes to favor photoprotection instead of photochemistry. An overall decrease in the protein content per cell is another potential strategy to decrease the overall Cu requirement of the cells [14]. Cu limitation modified the transcriptome of the chlorophyte *Chlamydomonas reinhardtii* with specific modification in the lipid pathways [17]. The authors from this study highlighted a desaturation of fatty acids associated to thykakoid membranes. To our knowledge, this was not assessed in diatoms despite their significance in global primary production and biogeochemical cycles [18].

The diatom *Pseudo-nitzschia* is distributed worldwide in coastal and oceanic areas including high nutrients low chlorophyll (HNLC) areas that are limited by Fe such as the subarctic pacific ocean [19]. Therefore, in these environments, *Pseudo-nitzschia* spp. might also be facing Cu limitations. However, *P. delicatissima* might have developed physiological strategies to face Cu limitation: under Cu limitation, *P. delicatissima* cells were able to maintain photosynthesis at the PSII step, unlike under Fe-limitation [20]. Although the rest of the photosynthetic chain was not studied and might have been partially impaired, the potential

physiological adaptation remains uncharacterized. The adaptation could occur at several levels, such as the Pcn/cytochrome c6 level but also at other photosynthetic or non-photosynthetic levels. Lelong and collaborators [20] observed significant variations in the overall lipid content and pigment fluorescence in Cu depleted cells but did not thoroughly studied these modifications that might reveal physiological adaptations. Overall, the strategies to cope with Cu limitations in *Pseudo-nitzschia* spp. and other marine microalgae remain largely unknown.

In this study, we aimed to further understand the physiological adaptation of the diatom *P. delicatissima* to Cu limitation and understand the specific roles of lipids. This research focused on the lipid composition (lipid classes and fatty acids) of the diatom, but also integrated the overall physiology (i.e. growth, cell size, C and N contents), especially the photophysiology (pigment composition and fluorescence of PSII) to better interpret the lipidomic responses.

Material and methods

Culture conditions

Batch cultures of the marine pennate diatom *Pseudo-nitzschia delicatissima* (strain Pd08RB, solitary species isolated in 2008 by Beatriz Beker in the Bay of Brest, France) were grown at 16°C in polycarbonate bottles. Species was determined after sequencing of the ITS-1 fragment, using PnAllR and PnAllF primers [21] and obtained sequences were aligned using GenBank. Cultures were grown under cool-white light (OSRAM) over a dark:light cycle of 12:12 h with an irradiance of 130 µmol photons m^{-2} s^{-1}. Although the cultures were naturally xenic and grown without antibiotics, the culture media (see below) were sterilized using a microwave [22]. Diatoms were pre-acclimated to each culture condition until their growth rate remained stable, and at least 20 generations were grown in the same conditions. Cultures were sampled in triplicates or quadriplicates (see below "Culture media") at mid-exponential phase of growth to maintain a pH lower than 8.5 and avoid CO_2 limitation, and at the same time of the day to avoid diel cycle variations.

Culture media

The culture medium consisted of artificial AQUIL seawater enriched with 300 µM nitrate, 10 µM phosphate, 100 µM silicate, 0.55 µg l^{-1} vitamin B_{12}, 0.5 µg l^{-1} biotin, 100 µg l^{-1} thiamin, 10 nM selenite and 100 nM molybdate [23]. The synthetic ocean water component (including all salts) and the macronutrients were passed through a Chelex 100 ion exchange resin to remove metal contaminants present in the chemicals [23]. The medium also contained a trace metal ion buffer system consisting of 100 µM ethylene diamine tetra acetic acid (EDTA), 50.3 nM Co, 79.7 nM Zn,121 nM Mn and 500 nM Fe. The buffer system generated free ion concentrations of Co, Zn, Mn and Fe of $10^{-10.88}$, $10^{-10.88}$, $10^{-8.27}$ and $10^{-19.2}$ M, respectively, at pH 8.1 according to the chemical equilibrium program MINEQL+ (version 4.62.3) for non-illuminated medium. In the replete medium ("Control"), 19.6 nM Cu were added, generating a

free Cu concentration of $10^{-13.9}$ M. In the Cu starvation treatment ("Cu-starved"), no Cu was added to the medium.

All bottles and apparatus were acid cleaned, and all manipulations were conducted within a sterile laminar flow hood equipped with a Teflon® bench using sterile and trace-metal clean techniques [24]. Cultures were run in triplicates for the Control and in quadriplicates for the Cu-starved condition.

Flow cytometric measurements

A flow cytometer FACScalibur (BD Biosciences, San Jose, CA USA) with an argon blue laser (488 nm) was used, with the same settings for all the duration of the experiment to allow comparison between treatments. Cell concentrations, morphological and physiological measurements of *P. delicatissima*, quantification of free-living bacteria associated to *P. delicatissima* and percentage of dead bacteria in the culture were assessed using flow cytometry [25]. As *Pseudo-nitzschia* spp. can form chains, cell concentrations were counted under microscope: each culture bottle was sampled in triplicate and the mean cell concentration is provided as an average.

Physiological measurements

Specific growth rate (μ, d^{-1}) was determined by linear regression of the natural log (cell concentration) versus time. Mortality of *P. delicatissima* was assessed by staining cultures with 0.1 µM of SYTOX Green (Molecular probes, Invitrogen, Eugene, Oregon, USA) for 30 minutes. More than 95 % of living cells could be observed in all cultures, which ensures that the physiological measurements were performed on living cells.

Bio-dilution

When cells divide, the cytoplasm and its content are divided between the two daughter cells. To assess the bio-dilution effect at steady state, the production rate (in arbitrary units per cell

per day (AU $cell^{-1}$ d^{-1}) of lipids can be calculated by multiplying their content (in AU $cell^{-1}$) by the acclimated specific growth rate (in d^{-1}) [20].

Bacteria

To estimate free-living bacteria concentration and viability, bacteria were analyzed after 15 min incubation with a final concentration of 1/10000 of the commercial solution of SYBR Green I (Molecular probes, Invitrogen, Eugene, Oregon, USA) and propidium iodide (PI, Sigma, St. Louis, MO, USA) at 10 µg ml^{-1} [25].

Cell volume

For each replicate, a minimum of 50 cells were photographed using a Leica microscope and the Axiovision imaging software. For each cell, cell length and cell width were measured. Cell volume was estimated using the following formulae of an ellipsoid [26]:

$$\text{Cell volume} = 1/6\pi * \text{length} * \text{width} * \text{width}.$$

Pulse-amplitude modulated (PAM) fluorimetry

Maximum quantum yield ($\Phi_{PSII} = (F_m-F_0)/F_m=F_v/F_m$), which is a measurement of the efficiency of the photosynthesis at the photosystem II (PSII) level, was measured using the AquaPen-C AP-C 100 fluorometer (Photo Systems Instruments, Czech Republic), where F_0 and F_m are respectively the minimum and maximum fluorescence of cells at 455 nm. The measurement of Φ_{PSII} was performed after 20 min of dark adaptation of the cells at 16°C. Chl a fluorescence induction transient (OJIP) curves were also performed on cultures to determine if Cu starvation modified the photosynthetic electron transport of *P. delicatissima*. Measurements were performed applying the internal protocol with blue light (455 nm). Complete dataset of fluorescent variables acquired during this study are available in the supplementary files.

The relative electron transport rate through PSII (rETRPSII) was calculated as:

$$rETR = \Phi_{PSII} * I$$

with I being the light intensity (comprised between 0 and 500 µmol photons m^{-2}).

Pigment composition and chlorophyll content

Duplicate samples from each culture bottle were filtered onto glass fiber filters (GF/F) for Chlorophyll *a* and pigments, and rinsed with artificial seawater previously sterilized by microwave. Filters for Chl *a* were immediately stored in glass tubes at -80 °C. Chl *a* was measured by fluorometry after extraction into 90% acetone [27].

Qualitative pigment composition was analyzed on pigments extracted from frozen cells (-80°C) by methanol using high-performance liquid chromatography (HPLC) according to the method described in [28], adapted from [29]. All the pigment standards were purchased from DHI (HØRSHOLM, Denmark). Duplicate cultures were used for pigment composition.

Cellular nitrogen and carbon

To determine the cellular C and N, culture samples were filtered as duplicates on a pre-combusted Whatman GF/F filter (450 °C for 4 hours) and rinsed with sterile artificial seawater containing no nutrient. The filters were dried at 60 °C overnight in pre-combusted glassware and stored until analysis using an elemental analyzer Thermo Fisher NA 2100 CN.

Lipids

About 9.10^6 cells from algal cultures were filtered on a GF/F filter. Boiling water was immediately added on filter to prevent lipid degradation through lipase activity. Total lipid extraction was performed placing the filter with algae in glass vials containing 6mL of a chloroform-methanol mixture (2/1, v/v). Lipid extract vials were then sealed under N_2 and stored at -20°C before further analyses. Lipid extracts were stored at -20°C under nitrogen (N2(g)) until analysis.

Lipid class composition analyses were performed by high-performance thin layer chromatography (HPTLC) using a CAMAG auto-sampler to spot the sample on HPTLC glass plates pre-coated with silica gel (Merck & Co., Ltd., Darmstadt, Germany). Neutral and polar

lipid classes were analyzed according to [30]. For polar and neutral lipid fatty acid (FA) analysis, an aliquot of the chloroform: methanol (2:1, v:v) extract was dried under $N_{2(g)}$ and then resuspended in chloroform:methanol (98:2, v:v) prior to neutral and polar lipid separation. Separation of neutral and polar lipids was realized by solid phase extraction [31]. Polar and neutral fractions were transesterified and the resulting fatty acid methyl esters (FAME) were analyzed and quantified by gas chromatography (GC-FID) according to the method from [31]. Lipid class and fatty acid standards were the same standards used in [31]. FA were expressed as percentage of total FA in polar and neutral lipids. The level of unsaturation was calculated from the % FA derived from the gas chromatographic data according to the equation:

$$Unsaturation\ level\ = \sum [\%\ of\ fatty\ acid\ \times number\ of\ double\ bond]$$

Statistics

Effects of Cu starvation on the physiological parameters of *P. delicatissima* were tested using T-tests with the software StatGraphics Plus (Manugistics, Inc, Rockville, MD, USA). The test of rank used was the Tukey test (variance homogeneity was first tested and confirmed for all the parameters). For all statistical results, a probability of $p < 0.05$ was considered significant.

Results

Flow cytometry and microscopy parameters

Specific growth rate

Control cultures grew at μ_{max} = 1.01 ± 0.01 d^{-1} (n=3), while Cu-starved cultures grew significantly slower ($p<0.01$), with a growth rate of 0.66 ± 0.01 d^{-1} (n=4) (Table 1). Cu-starved cells had a significantly higher length and width ($p<0.01$); leading to a cell volume 24% higher than control cells ($p<0.01$) (Table 1).

Table 1: Physiologic and morphometric parameters of P. delicatissima in the control and under Cu starved conditions. Results are expressed as mean ± standard deviation. Statistical differences between the two treatments are indicated as followed "NS" not significant, "" 0.01 < p-value < 0.05, "**" 0.001 < p-value < 0.01.*

	Control	Cu-starved	p-value
μ (day^{-1})	1.01 ± 0.01	0.66 ± 0.01	**
Length (μm)	16.83 ± 0.17	17.66 ± 0.10	**
Width (μm)	2.99 ± 0.05	3.25 ± 0.01	**
Volume (μm^3)	79.2 ± 1.7	98.4 ± 0.5	**
% of dead cells	2.6 ± 2.5	0.9 ± 0.3	NS
Ratio Bacteria/*P. delicatissima*	0.70 ± 0.09	2.4 ± 0.3	**

Dead and active cells

Cu starvation did not have any effect on the mortality of *P. delicatissima*. Less than 3% of the cells were considered dead (i.e. cells stained by SYTOX green) in the control and in Cu-starved conditions (Table 1), which showed no significant difference.

Bacteria

There were significantly ($p<0.01$) more bacteria in the Cu-starved cultures per *P. delicatissima* cell (Table 1). The biovolume of bacteria was estimated to represent less than 4% of microalgal biomass, therefore, the contribution of bacteria to the biomass was considered as negligible in our biochemical analyses (C, N and lipid contents).

Photosynthetic parameters

Photosystem II activity

The maximum PSII quantum yield (Figure 1A) was significantly lower ($p<0.01$) in Cu-starved cells (0.58 ± 0.01) than in control cells (0.62 ± 0.00). Light curves (Figure 1B) show that Cu-starved cells exhibited lower $rETR_{PSII}$ than control cells, regardless of the value of the light pulse (20 to 500 µmol photons.m^{-2}).

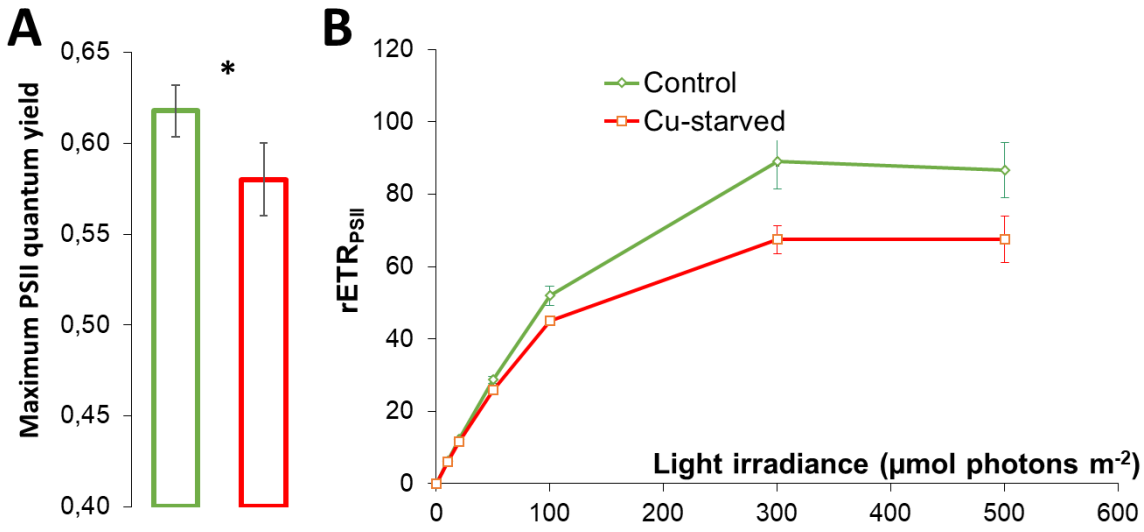

Figure 1: Effect of Cu depletion on photosystem II (PSII) of P. delicatissima. A) Maximum PSII quantum yield in the control (green) and in the Cu-starved conditions (red). Statistical differences between the two treatments are indicated as followed "NS" not significant, "" 0.01 < p-value < 0.05, "**" 0.001 < p-value < 0.01. B) Relative electron transport through PSII ($rETR_{PSII}$) in the control (green) and in the Cu-starved conditions (red). Error bars represent the standard deviation. Other measured photosynthetic parameters can be found in Supplementary files.*

Cellular nitrogen and carbon

Cu-starved cells contained significantly more C ($p<0.01$) and N ($p<0.05$) than the cells in replete conditions (Table 2). When corrected to cell volume, the difference in C and N contents between the two conditions were not significant anymore ($p>0.05$). However, the C/N ratio was significantly higher by 20% in Cu-starved cells than in cells from the control ($p<0.01$).

Table 2: Cellular C and N content of P.delicatissima in the control and in the Cu-starved conditions. Results are expressed as mean ± standard deviation. Statistical differences between the two conditions are indicated as followed "NS" not significant, "" 0.01 < p-value < 0.05, "**" 0.001 < p-value < 0.01.*

	Control	Cu-starved	p-value
C (pmol cell^{-1})	0.73 ± 0.03	1.0 ± 0.1	**
C (mol dm^{-3} of cells)	9.6 ± 0.5	11.0 ± 0.9	NS
N (pmol cell^{-1})	0.12 ± 0.01	0.15 ± 0.01	*
N (mol dm^{-3} of cells)	1.6 ± 0.1	1.6 ± 0.1	NS
C/N	5.9 ± 0.1	7.0 ± 0.3	**

Pigment composition and chlorophyll content

The chlorophyll content was significantly ($p<0.01$) increased by 54% in Cu-starved cells as compared to control cells (Table 3). When corrected to cell volume, chlorophyll *a* content of Cu-starved cells was still significantly higher ($p<0.05$), but only by 23%. The overall pigment composition of *P. delicatissima* was affected by Cu starvation with an increase in the proportion of three pigments (Chlorophyllide a, Fucoxanthine, Diadinoxanthine) and a decrease in the proportion of two pigments (Chlorophyll C2, Chlorophyll a).

Tableau 3: Pigment composition and chlorophyll content of P. delicatissima in the control and in the Cu-starved conditions. Results are expressed as mean ± standard deviation. Statistical differences could not be tested for the % of each pigment class (N = 2). Statistical differences in the chlorophyll (Chl) content are indicated as followed "NS" not significant, "" 0.01 < p-value < 0.05, "**" 0.001 < p-value < 0.01.*

		Control	Cu-starved	Trend or p-value
%	Chlorophyll C3	4.4 ± 0.1	4.3 ± 0.4	≈
	Chlorophyll C2	7.6 ± 0.1	3.7 ± 0.8	↘
	chlorophyllid a	3.0 ± 1.0	10.1 ± 3.3	↗
	Fucoxanthin	39.6 ± 2.2	54.7 ± 2.3	↗
	Violaxanthin	0.2 ± 0.0	0.0 ± 0.0	≈
	Diadinoxanthin	2.7 ± 0.1	5.4 ± 1.1	↗
	Diatoxanthin	0.2 ± 0.0	0.2 ± 0.0	≈
	Chlorophyll a	41.2 ± 2.8	20.6 ± 3.5	↘
	b_carotene	1.1 ± 0.0	1.1 ± 0.1	≈
	Chl a (pg cell^{-1})	0.15 ± 0.01	0.23 ± 0.02	**
	Chl a content (pg/µm^3 cell)	1.89 10^{-3} ± 0.10 10^{-3}	2.35 10^{-3} ± 0.19 10^{-3}	*

Chl: Chlorophyll

Lipids

Lipid classes

Copper starvation induced a significant increase in the content and productivity of lipids by *P. delicatissima* (Table 4). The composition of lipid classes was also affected with significant decreases in the proportions of sulfoquinovosyl diacylglycerol (SQDG) ($P<0.05$), phosphatidylglycerine (PG) ($P<0.001$), free fatty acids (FFA) ($P<0.01$) and significant increases in the proportions of digalactosyl diacylglycerol (DGDG) ($P<0.001$) and triglycerides (TAG) ($P<0.01$).

Table 4: Total (membrane and storage) lipid classes composition and productivity of P. delicatissima in the control and in the Cu-starved conditions. Results are expressed as mean ± standard deviation. Statistical differences between 20Cu and 0Cu are indicated as followed "NS" not significant, "" 0.01 < p-value < 0.05, "**" 0.001 < p-value < 0.01, "***" p-value < 0.001.*

		Control	Cu-starved	p-value
Membranes	% SQDG	19.8 ± 0.7	17.7 ± 0.7	*
	% DGDG	5.9 ± 0.2	10.2 ± 0.4	***
	% MGDG	44.1 ± 2.2	45.4 ± 1.3	NS
	% PG	18.1 ± 0.1	11.9 ± 0.9	***
	% PE	0 ± 1	1.9 ± 0.8	NS
	% phytosterols	12.4 ± 0.8	13.0 ± 1.5	NS
	Content (pg cell^{-1})	2438 ± 210	3691 ± 244	***
	Productivity (pg cell^{-1} day^{-1})	2461 ± 189	2450 ± 150	NS
	Content/volume of cells (pg µm^{-3})	32 ± 3	71 ± 9	*
Storage	% FFA	27.5 ± 3.0	6.1 ± 6.7	**
	% TAG	72.5 ± 3.0	93.9 ± 6.7	**
	Content (pg cell^{-1})	55 ± 12	1648 ± 302	**
	Productivity (pg cell^{-1} day^{-1})	55 ± 12	1093 ± 192	*
	Content/volume of cells (pg µm^{-3})	0. 7 ± 0.2	17 ± 3	*
Total	Content (pg cell^{-1})	2493 ± 215	5339 ± 540	***
	Productivity (pg cell^{-1} day^{-1})	2516 ± 194	3543 ± 337	**

SQDG: sulfoquinovosyl-diacylglycerol, DGDG: digalactosyl-diacylglycerol, MGDG: monogalactosyl-diacylglycerol, PG: phosphatidylglycerine, PE: phosphatidylethanolamine, FFA: free fatty acids, TAG: triglycerides

Fatty acid (FA) composition and phytosterols

Changes in lipid classes occurred alongside significant modifications in polar (Figure 2A-B) and neutral (Figure 2C-D) FA compositions. Cu starvation induced a significant increase in the proportions of polar 16:1n-7, 18:1n-7, 18:1n-5 and neutral 16:1n-7, 16:2n-4 FA, while the proportions of neutral 16:0, 18:0 and 22:6n-3 FA significantly decreased. The proportions of the two phytosterols, were also significantly modified by the unavailability of Cu in the culture media, with a decreased cholesterol, while the 24-methylenecholesterol was increased (Figure 3).

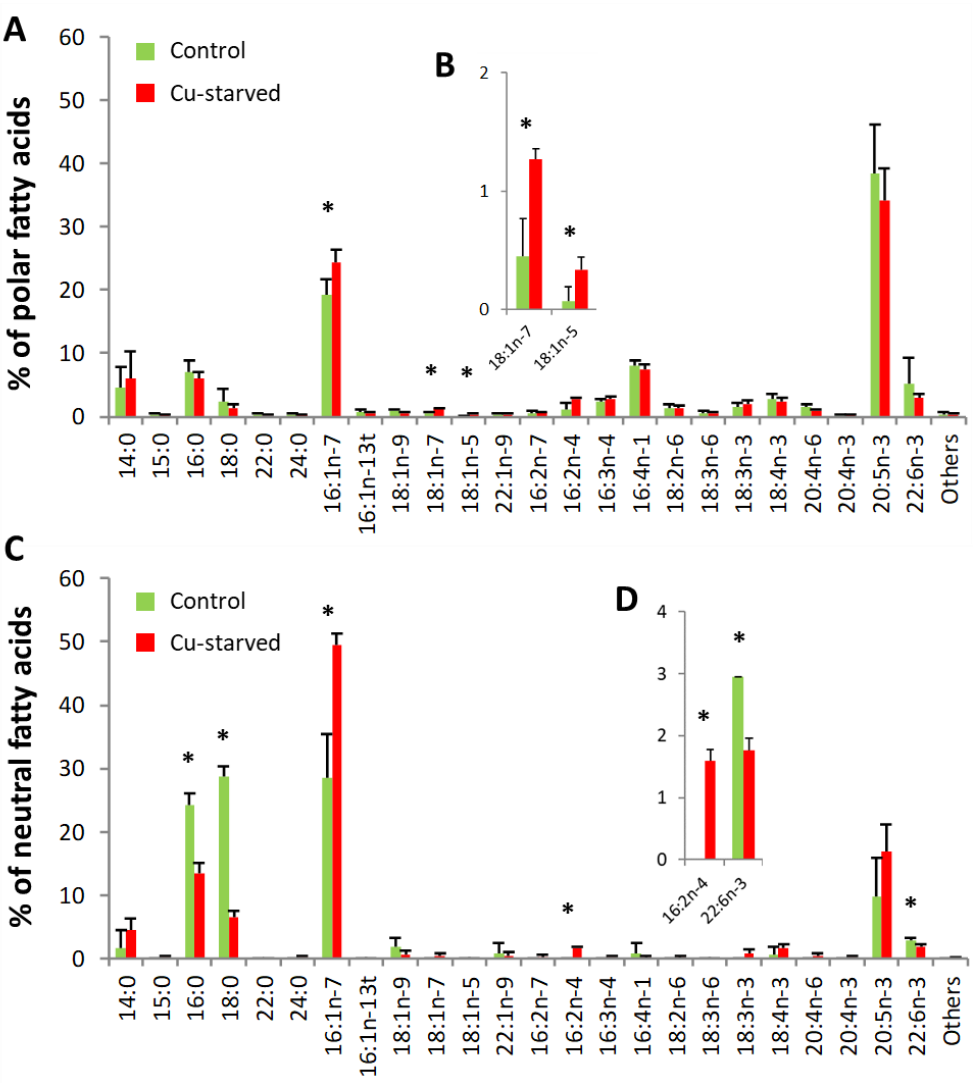

Figure 2: Composition of A-B) polar (membranes) fatty acids (FA), C-D) neutral (storage) FA of P. delicatissima in the control (green) and in the Cu-starved conditions (red). Zooms of the significant differences of FA in small proportions are given in B) and D). Results are expressed as mean ± standard deviation. Statistical differences between the two conditions are indicated as followed "NS" not significant, "" 0.01 < p-value < 0.05, "**" 0.001 < p-value < 0.01, "***" p-value < 0.001.*

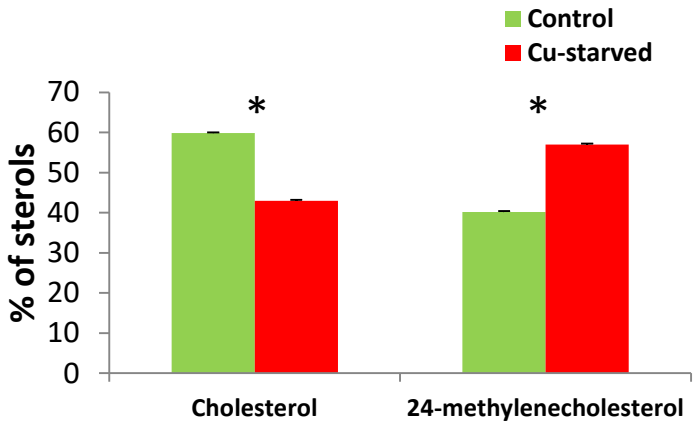

Figure 3: Composition of phytosterols of P. delicatissima in the control and in the Cu starved conditions. Results are expressed as mean ± standard deviation. Statistical differences between the two conditions are indicated as followed "NS" not significant, "" 0.01 < p-value < 0.05, "**" 0.001 < p-value < 0.01, "***" p-value < 0.001.*

Discussion

Physiological effects of Cu starvation. Copper starvation significantly affected the overall physiology of *P. delicatissima* by inhibiting cell growth, increasing cell size, cellular C and N content and modifying the overall biochemistry (C/N ratio, lipids, pigments) of the diatoms. Yet it did not induce any excess mortality nor totally suppress growth, indicating that the structural modifications might have played a role in the adaptation to Cu starvation.

Photophysiology of Cu starvation. While literature reports contrasting effects of Cu-limitation on the physiological/morphological responses, including photophysiology, of microalgae [14,32,33], the photosystem II (PSII) of *P. delicatissima* was only slightly affected by Cu starvation which corroborates with results from a previous study [20]. However, the relative electron flow at PSII ($rETR_{PSII}$) was significantly decreased, as similarly observed in *T. oceanica* under Cu limitation [4]. While the maximum PSII quantum yield only highlights one step of the photosynthetic chain, the relative electron flow at PSII ($rETR_{PSII}$) provides a better picture of photosynthesis. The inhibition of $rETR_{PSII}$ of *P. delicatissima* observed in the present study highlights a blockage of the electron flow in the photosynthetic chain upstream or downstream of PSII, at the Calvin-Benson-Bassham cycle for instance. Between the PSII

and the cytochrome b6-f, the photosynthetic chain of diatoms can contain plastocyanin (PCn), a Cu-containing protein carrying electrons. The decrease in $rETR_{PSII}$ suggests that *P. delicatissima* may contain some PCn, which functioning might have been limited by Cu. A lack of PCn electron carrier might have resulted in a bottleneck limiting the electron flow. However, the $rETR_{PSII}$ was not totally suppressed, and cells were still able to grow, suggesting that cells were able to adapt to Cu starvation stress. Some algae contain both PCn and cytochrome c6 and/or can shift from Cu-containing PCn to Fe-containing cytochrome c6 when Cu is limiting [14]. This is for example the case of coastal diatoms that are rarely facing metal limitations and can synthesize both Cu-containing PCn and Fe-containing cytochrome c6 [4,34]. It is likely that the coastal strain of *P. delicatissima* used in our study and in [20] can synthesize cytochrome c6 in addition to PCn. This would also explain the growth rate and maximum PSII QY inhibitions of *P. delicatissima* observed under Fe limitation [20]. Therefore, under Cu-starved, *P. delicatissima* might have shifted from PCn to more cytochrome c6 electron carriers to counteract the bottleneck in the electron flow and compensate Cu starvation. While in the literature the photosynthetic chain received most of the attention, Cu is a constituent of several proteins and its unavailability might have inhibited other metabolic pathways downstream of the photosynthetic chain that might have impaired the electron transport. Quantification of PCn and Cyt c6 in *P. delicatissima* is required to fully understand the inhibition of photosynthesis.

Rearrangement of photosynthetic membranes. Cu starvation induced a bottleneck in the photosynthetic electron flow that increases the excitation in the pigment antennae and favors the production of ROS and photodamages. However, under the light conditions of this experiment, the maximum PSII QY of *P. delicatissima* was only slightly decreased, highlighting some adaptation of the photosynthetic apparatus. Among the mechanisms to cope with the lack of Cu, the oceanic diatom *Thalassiosira oceanica* rearranges its light harvesting

368 complexes (LHCs) from light harvesting to photoprotection under Cu limitation [14].
369 Furthermore the proportion of pigments (fucoxanthin, Chl c,a and Ddx) associated to energy
370 dissipation in fucoxanthin chlorophyll a/c-binding protein (FCP) [35], the protein forming
371 LHC, was significantly increased in the absence of Cu. Overall, this rearrangement results in
372 an increase in a better dissipation of energy coming from the electron flow bottleneck.
373 Rearrangement of LHCs is accompanied with biochemical modifications of the membranes.
374 In our experiments, LHCs were not quantified, but the biochemistry of membranes
375 highlighted a rearrangement of *P. delicatissima* photosynthetic chain with an increase in lipid
376 classes associated to light harvesting complex II (LHCII) such as digalactosyl diacylglycerol
377 (DGDG) and phosphatidylglycerine (PG)[36]. The modifications in the proportion of lipid
378 classes as observed with SQDG, DGDG and PG might be responsible for the modification in
379 polar FA composition. The modifications in these FA might also come from modifications of
380 FA within specific lipid classes: in the green algae *Chlamydomonas reinharditii*, Cu
381 limitation induced a modification of galactolipid composition and a specific desaturation of
382 FA from DGDG [17]. All these modifications in the lipid and pigment compositions suggests
383 a potential increase in LHCs in *P. delicatissima* in response to Cu starvation. The
384 photophysiological responses of the strain of *P. delicatissima* from our study are somewhat
385 similar from the photophysiological responses of *T. oceanica* [14]: the rearrangement of the
386 photosynthetic apparatus in *T. oceanica* leads to the increase of LHCs associated with an
387 increase in NPQ. The biochemical modification of *P. delicatissima* photosynthetic
388 membranes could support the hypothesis of a restructuration of LHC towards photoprotection,
389 although no significant increase in NPQ was measured, even at saturating light intensities (>
390 300 µmol photons m^{-2}).
391 *Cu-starvation modifies the overall lipid metabolism*. Under the light conditions used
392 for culture growth (130 µmol photon m^{-2}), while the photosynthesis was only slightly

393 inhibited, the microalgal growth was significantly inhibited by 35%. Therefore, the energy
394 produced by photosynthesis that was not used for growth had to be somehow used or stored.
395 “Excess” energy storage can be stored under several forms in microalgae [37,38]. The C/N
396 ratio was higher in Cu-starved cells, and this increase could be linked to the increase in
397 polysaccharides and lipids, more likely than an increase in the production of proteins. When
398 microalgae are not able to divide, they can store their energy in the form of lipids [39], as
399 observed with the daily production rate of storage lipids that significantly increased in Cu-
400 starved conditions in this study and a previous one [20]. Accumulation of triacylglycerol
401 (TAG) could also be an effective way to prevent photooxidative damages from altered *P.*
402 *delicatissima* photosynthetic chain. TAG can be a sink for electrons of photosynthetic
403 reactions [40]. Once favorable conditions return, the energy stored under the form of TAG
404 can also easily be mobilized for cells to divide. The drastic increase in lipid content might
405 explain the higher C/N ratio under Cu starvation, although it could also be partially attributed
406 to a decrease of the protein content per cell: a strategy used by the diatom *Thalassiosira*
407 *oceanica* to decrease cellular Cu demand [14].
408 Beside the overall increase in neutral lipids, neutral FA composition (exclusively composed of
409 FFA and triglycerides) was also modified underlying physiological adaptations. The
410 percentage of FA with 16 and 18 carbons were the most affected by Cu starvation. Within the
411 neutral FA, the proportion of the saturated FA 16:0 and 18:0 were the most affected with a
412 decrease in absence of Cu. Neutral 16:0 were likely converted in 16:1n-7 (the main FA form
413 for triglycerides; Li et al. 2014) and in small proportions of 16:2n-4, while 18:0 were likely
414 mobilized for the formation of 18:1n-7 and 18:1n-5 as their proportion significantly increased
415 in polar and neutral lipids. Eventually, the increase in 18:1n-7 might originate from the
416 elongation 16:1n-7 as suggested by [41]. In the green microalgae *Dunaliella*, Cu limitation
417 induced a similar desaturation of FA with 16 and 18 carbons [33]. Modification of neutral FA

metabolism mainly reflects a storage of energy under the form of the mono-unsaturated FA 16:1n-7. Other modifications may also originate from the *de-novo* synthesis of FA to meet the demand of new membrane lipids, especially for photosynthetic membranes to adapt to Cu starvation, or to replace FA peroxidated by ROS coming from photooxidation [13].

Cu starvation induced a shift in phytosterols. Cu starvation modified proportions of phytosterols, the ratio cholesterol/24-methylenecholesterol was reversed under Cu starvation with an increase of 24-methylenecholesterol. Cholesterol and 24-methylenecholesterol only differ from a doubled bond within the radical chain of the molecule. While the role of phytosterols under Cu starvation is unknown, sterols have many roles such as for example regulation of plasmic membrane fluidity and permeability, signal transduction, modulation of enzyme activity, or precursors of secondary metabolites [42,43]. Among the roles that could be related to Cu starvation, sterols are essential for the functioning of cellular membranes, photosynthesis [44,45] and are likely involve in FA biosynthesis as highlighted in *Nannochloropsis oceanica* [45]. However, their precise role during Cu starvation remains to be elucidated in further analyses.

Conclusion

In the present study we investigated the physiological responses of a strain of *P. delicatissima* to Cu starvation with a special interest for biochemical changes (lipids and pigments) in relation with the overall physiology. Despite Cu starvation, the diatom was able to grow and maintain some photosynthetic activity. The lipidomic approach revealed two physiological adaptations to counteract Cu starvation a) membrane rearrangements and b) energetic adaptation. Rearrangement of membrane lipids was highlighted by modifications in phytosterol composition and increases in lipids and pigments associated with photoprotective responses. These responses must have helped maintaining the photosynthetic efficiency and mitigate damages on photosystem II. In parallel, the energy captured by photosynthesis was partially diverted from microalgal growth to lipid production. Cells were bigger and stored

energy as triglycerides (with 16:1n-7 being the main FA stored). The stored energy could be mobilized for cells to resume growth when conditions become favorable (i.e., when Cu starvation is relieved). This modification in C allocation may have ecological relevance. The drastic increase in lipids (TAG content was 40 times higher in Cu-starved cells), other things being equal, may change the overall density of the cells [46] and, therefore, their position in the water column [47,48]. This effect on buoyancy could be reinforced or counteracted by the accumulation of other cell components. In diatoms, an appreciable contribution to cell density is given by the silica frustule [48]. However, for this specific strain of *P. delicatissima* (Pd08RB), Cu starvation did not affect the biogenic silica content of the cells in a previous study [49] and would not counteract the buoyancy effect of lipids. Although this study revealed an adaptation strategy to Cu starvation, one has to be cautious and should not generalize these results to different strains. Different strains from the same species can have contrasting physiological responses to Cu stress [14] and further study should investigate the strain variability of *P. delicatissima* responses.

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