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# 1 **Physiological adaptation of the diatom *Pseudo-nitzschia delicatissima* under** 2 **copper starvation**

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9  
10 Keywords: bacillariophyta, copper starvation, physiology, membranes, lipid metabolism,  
11 energy storage, photosynthesis, photosystem II

## 12 13 **Dataset**

14 The dataset is publicly available at: <https://www.seanoe.org/data/00833/94472/>

## 15 16 **Abstract**

17 In the open ocean and particularly in iron (Fe)-limited environment, copper (Cu) deficiency  
18 might limit the growth of phytoplankton species. Cu is an essential trace metal used in  
19 electron-transfer reactions, such as respiration and photosynthesis, when bound to specific  
20 enzymes. Some phytoplankton species, such as the diatom *Pseudo-nitzschia* spp. can cope  
21 with Cu starvation through adaptative strategies. In this study, we investigated the  
22 physiological strategies of the marine diatom *P. delicatissima* against Cu starvation.

23 Compared to the control, Cu starvation inhibited growth by 35%, but did not induce any  
24 excess mortality. Despite the bottleneck measured in the electron flow of the photosynthetic  
25 chain, cells of *P. delicatissima* conserved their photosynthesis ability. This photosynthesis  
26 maintenance was accompanied by structural changes of membranes, where pigments and lipid  
27 composition were strongly modified. Diatoms also strongly modified their metabolism, by  
28 redirecting their C allocation to energy storage under the form of triglycerides. By  
29 maintaining essential metabolic functions and storing energy under the form of lipids, these

30 physiological adaptations might be a strategy enabling this diatom to later bloom under the  
31 return of favorable nutritional condition.

32

33

## 34 **Introduction**

35

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

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

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

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

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

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

97

## 98 **Material and methods**

### 99 **Culture conditions**

100 Batch cultures of the marine pennate diatom *Pseudo-nitzschia delicatissima* (strain Pd08RB,  
101 solitary species isolated in 2008 by Beatriz Beker in the Bay of Brest, France) were grown at  
102 16°C in polycarbonate bottles. Species was determined after sequencing of the ITS-1  
103 fragment, using PnAllR and PnAllF primers [21] and obtained sequences were aligned using  
104 GenBank. Cultures were grown under cool-white light (OSRAM) over a dark:light cycle of  
105 12:12 h with an irradiance of 130  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Although the cultures were naturally  
106 xenic and grown without antibiotics, the culture media (see below) were sterilized using a  
107 microwave [22]. Diatoms were pre-acclimated to each culture condition until their growth rate  
108 remained stable, and at least 20 generations were grown in the same conditions. Cultures were  
109 sampled in triplicates or quadruplicates (see below “Culture media”) at mid-exponential phase  
110 of growth to maintain a pH lower than 8.5 and avoid CO<sub>2</sub> limitation, and at the same time of  
111 the day to avoid diel cycle variations.

112

### 113 **Culture media**

114 The culture medium consisted of artificial AQUIL seawater enriched with 300  $\mu\text{M}$  nitrate, 10  
115  $\mu\text{M}$  phosphate, 100  $\mu\text{M}$  silicate, 0.55  $\mu\text{g l}^{-1}$  vitamin B<sub>12</sub>, 0.5  $\mu\text{g l}^{-1}$  biotin, 100  $\mu\text{g l}^{-1}$  thiamin,  
116 10 nM selenite and 100 nM molybdate [23]. The synthetic ocean water component (including  
117 all salts) and the macronutrients were passed through a Chelex 100 ion exchange resin to  
118 remove metal contaminants present in the chemicals [23]. The medium also contained a trace  
119 metal ion buffer system consisting of 100  $\mu\text{M}$  ethylene diamine tetra acetic acid (EDTA), 50.3  
120 nM Co, 79.7 nM Zn, 121 nM Mn and 500 nM Fe. The buffer system generated free ion  
121 concentrations of Co, Zn, Mn and Fe of  $10^{-10.88}$ ,  $10^{-10.88}$ ,  $10^{-8.27}$  and  $10^{-19.2}$  M, respectively, at  
122 pH 8.1 according to the chemical equilibrium program MINEQL+ (version 4.62.3) for non-  
123 illuminated medium. In the replete medium (“Control”), 19.6 nM Cu were added, generating a

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

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

130

### 131 **Flow cytometric measurements**

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

140

### 141 *Physiological measurements*

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

147

### 148 *Bio-dilution*

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

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

153

#### 154 *Bacteria*

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

159

#### 160 **Cell volume**

161 For each replicate, a minimum of 50 cells were photographed using a Leica microscope and  
162 the Axiovision imaging software. For each cell, cell length and cell width were measured.

163 Cell volume was estimated using the following formulae of an ellipsoid [26]:

$$164 \text{Cell volume} = 1/6\pi * \text{length} * \text{width} * \text{width}.$$

165

#### 166 **Pulse-amplitude modulated (PAM) fluorimetry**

167 Maximum quantum yield ( $\Phi_{\text{PSII}} = (F_m - F_0)/F_m = F_v/F_m$ ), which is a measurement of the  
168 efficiency of the photosynthesis at the photosystem II (PSII) level, was measured using the  
169 AquaPen-C AP-C 100 fluorometer (Photo Systems Instruments, Czech Republic), where  $F_0$   
170 and  $F_m$  are respectively the minimum and maximum fluorescence of cells at 455 nm. The  
171 measurement of  $\Phi_{\text{PSII}}$  was performed after 20 min of dark adaptation of the cells at  $16^\circ\text{C}$ . Chl  
172 a fluorescence induction transient (OJIP) curves were also performed on cultures to determine  
173 if Cu starvation modified the photosynthetic electron transport of *P. delicatissima*.

174 Measurements were performed applying the internal protocol with blue light (455 nm).

175 Complete dataset of fluorescent variables acquired during this study are available in the  
176 supplementary files.

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

178 
$$rETR = \Phi_{PSII} * I$$

179 with I being the light intensity (comprised between 0 and 500  $\mu\text{mol photons m}^{-2}$ ).

180

### 181 **Pigment composition and chlorophyll content**

182 Duplicate samples from each culture bottle were filtered onto glass fiber filters (GF/F) for

183 Chlorophyll *a* and pigments, and rinsed with artificial seawater previously sterilized by

184 microwave. Filters for Chl *a* were immediately stored in glass tubes at -80 °C. Chl *a* was

185 measured by fluorometry after extraction into 90% acetone [27].

186 Qualitative pigment composition was analyzed on pigments extracted from frozen cells (-

187 80°C) by methanol using high-performance liquid chromatography (HPLC) according to the

188 method described in [28], adapted from [29]. All the pigment standards were purchased from

189 DHI (HØRSBOLM, Denmark). Duplicate cultures were used for pigment composition.

### 190 **Cellular nitrogen and carbon**

191 To determine the cellular C and N, culture samples were filtered as duplicates on a pre-

192 combusted Whatman GF/F filter (450 °C for 4 hours) and rinsed with sterile artificial

193 seawater containing no nutrient. The filters were dried at 60 °C overnight in pre-combusted

194 glassware and stored until analysis using an elemental analyzer Thermo Fisher NA 2100 CN.

### 195 **Lipids**

196 About  $9 \cdot 10^6$  cells from algal cultures were filtered on a GF/F filter. Boiling water was

197 immediately added on filter to prevent lipid degradation through lipase activity. Total lipid

198 extraction was performed placing the filter with algae in glass vials containing 6mL of a

199 chloroform-methanol mixture (2/1, v/v). Lipid extract vials were then sealed under N<sub>2</sub> and

200 stored at -20°C before further analyses. Lipid extracts were stored at -20°C under nitrogen

201 (N<sub>2</sub>(g)) until analysis.

202 Lipid class composition analyses were performed by high-performance thin layer

203 chromatography (HPTLC) using a CAMAG auto-sampler to spot the sample on HPTLC glass

204 plates pre-coated with silica gel (Merck & Co., Ltd., Darmstadt, Germany). Neutral and polar

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

$$215 \quad \text{Unsaturation level} = \sum [\% \text{ of fatty acid} \times \text{number of double bond}]$$

## 216 **Statistics**

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

221

222

## 223 **Results**

### 224 **Flow cytometry and microscopy parameters**

#### 225 *Specific growth rate*

226 Control cultures grew at  $\mu_{\max} = 1.01 \pm 0.01 \text{ d}^{-1}$  (n=3), while Cu-starved cultures grew  
227 significantly slower ( $p < 0.01$ ), with a growth rate of  $0.66 \pm 0.01 \text{ d}^{-1}$  (n=4) (Table 1). Cu-  
228 starved cells had a significantly higher length and width ( $p < 0.01$ ); leading to a cell volume  
229 24% higher than control cells ( $p < 0.01$ ) (Table 1).

230

231 *Table 1: Physiologic and morphometric parameters of P. delicatissima in the control and under Cu starved conditions.*

232 *Results are expressed as mean  $\pm$  standard deviation. Statistical differences between the two treatments are indicated as*

233 *followed "NS" not significant, "\*"  $0.01 < p\text{-value} < 0.05$ , "\*\*\*"  $0.001 < p\text{-value} < 0.01$ .*

	Control	Cu-starved	p-value
$\mu$ (day <sup>-1</sup> )	1.01 $\pm$ 0.01	0.66 $\pm$ 0.01	**
Length ( $\mu\text{m}$ )	16.83 $\pm$ 0.17	17.66 $\pm$ 0.10	**
Width ( $\mu\text{m}$ )	2.99 $\pm$ 0.05	3.25 $\pm$ 0.01	**
Volume ( $\mu\text{m}^3$ )	79.2 $\pm$ 1.7	98.4 $\pm$ 0.5	**
% of dead cells	2.6 $\pm$ 2.5	0.9 $\pm$ 0.3	NS
Ratio			
Bacteria/ <i>P. delicatissima</i>	0.70 $\pm$ 0.09	2.4 $\pm$ 0.3	**

234

235

236

#### 237 *Dead and active cells*

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

#### 241 *Bacteria*

242 There were significantly ( $p < 0.01$ ) more bacteria in the Cu-starved cultures per *P.*  
243 *delicatissima* cell (Table 1). The biovolume of bacteria was estimated to represent less than  
244 4% of microalgal biomass, therefore, the contribution of bacteria to the biomass was  
245 considered as negligible in our biochemical analyses (C, N and lipid contents).

246

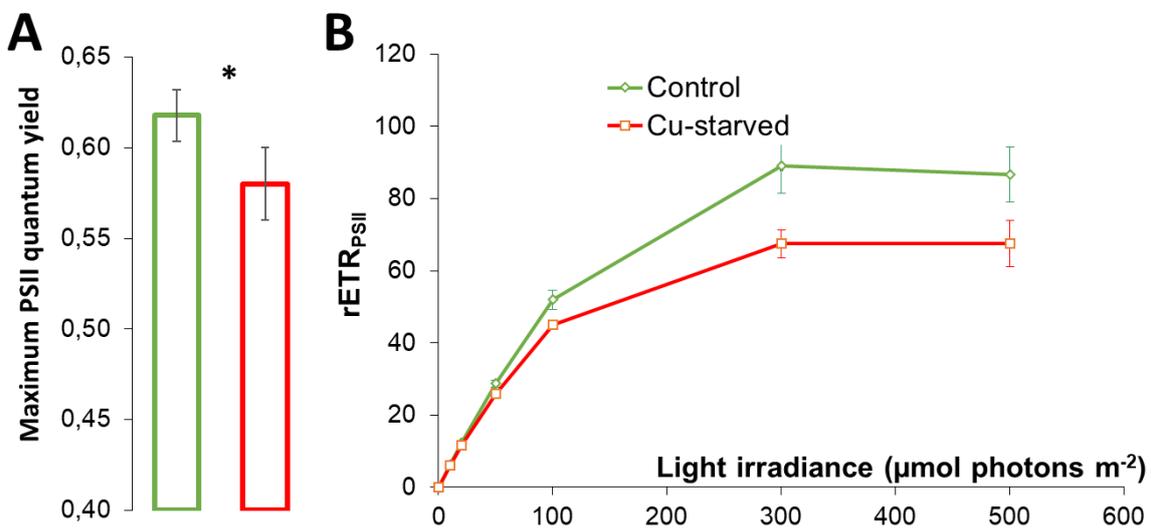
247

## 248 **Photosynthetic parameters**

### 249 *Photosystem II activity*

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

254



255

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

### 261 *Cellular nitrogen and carbon*

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

267

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

271

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

272

273

#### 274 *Pigment composition and chlorophyll content*

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

281

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

286

	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 <sup>-1</sup> )	0.15 ± 0.01	0.23 ± 0.02	**
Chl a content (pg/μm <sup>3</sup> cell)	1.89 10 <sup>-3</sup> ± 0.10 10 <sup>-3</sup>	2.35 10 <sup>-3</sup> ± 0.19 10 <sup>-3</sup>	*

Chl: Chlorophyll

287

288

## 289 **Lipids**

290 *Lipid classes*

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

297

298 *Table 4: Total (membrane and storage) lipid classes composition and productivity of P. delicatissima in the control and in*  
 299 *the Cu-starved conditions. Results are expressed as mean ± standard deviation. Statistical differences between 20Cu and 0Cu*  
 300 *are indicated as followed “NS” not significant, “\*” 0.01 < p-value < 0.05, “\*\*\*” 0.001 < p-value < 0.01, “\*\*\*\*” p-value <*  
 301 *0.001.*

302

		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 <sup>-1</sup> )	2438 ± 210	3691 ± 244	***
	Productivity (pg cell <sup>-1</sup> day <sup>-1</sup> )	2461 ± 189	2450 ± 150	NS
	Content/volume of cells (pg μm <sup>-3</sup> )	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 <sup>-1</sup> )	55 ± 12	1648 ± 302	**
	Productivity (pg cell <sup>-1</sup> day <sup>-1</sup> )	55 ± 12	1093 ± 192	*
	Content/volume of cells (pg μm <sup>-3</sup> )	0.7 ± 0.2	17 ± 3	*
Total	Content (pg cell <sup>-1</sup> )	2493 ± 215	5339 ± 540	***
	Productivity (pg cell <sup>-1</sup> day <sup>-1</sup> )	2516 ± 194	3543 ± 337	**

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

303

304

305

### 306 *Fatty acid (FA) composition and phytosterols*

307 Changes in lipid classes occurred alongside significant modifications in polar (Figure 2A-B)

308 and neutral (Figure 2C-D) FA compositions. Cu starvation induced a significant increase in

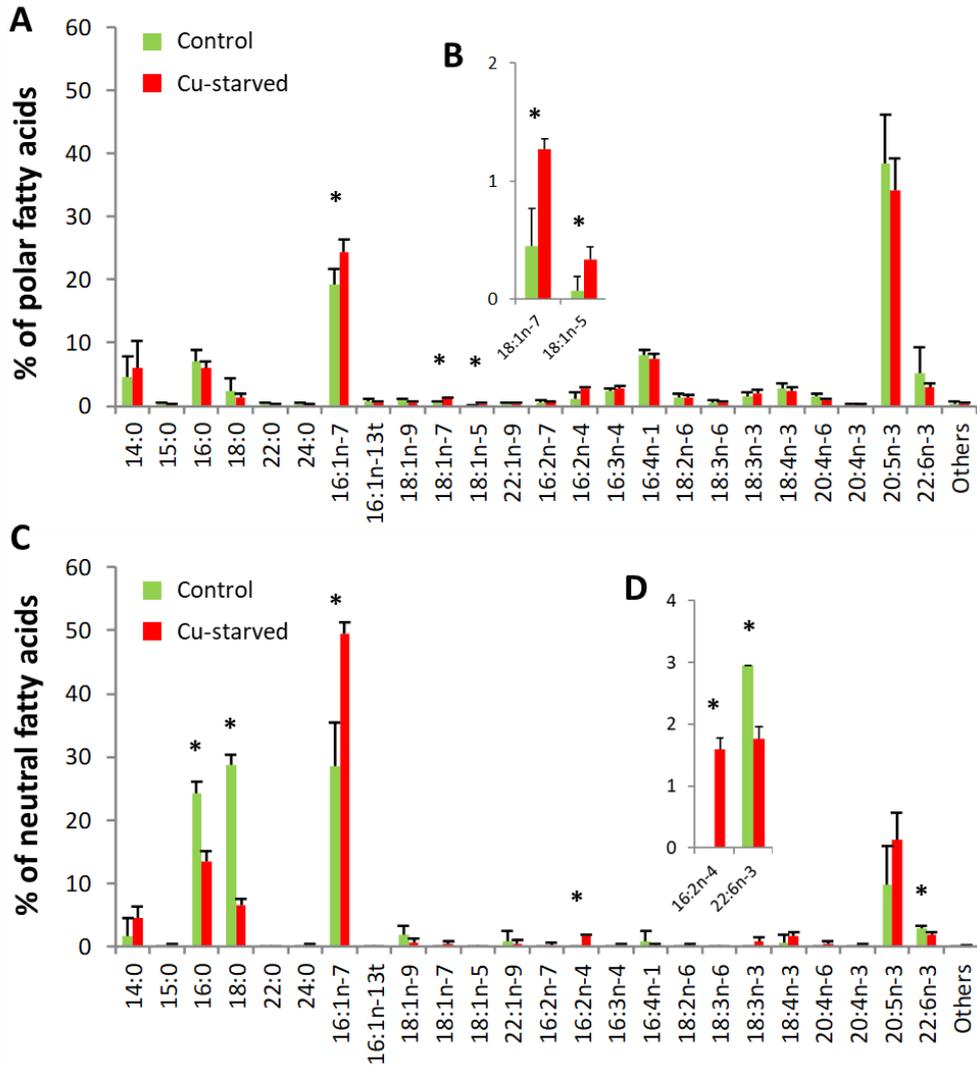
309 the proportions of polar 16:1n-7, 18:1n-7, 18:1n-5 and neutral 16:1n-7, 16:2n-4 FA, while the

310 proportions of neutral 16:0, 18:0 and 22:6n-3 FA significantly decreased. The proportions of

311 the two phytosterols, were also significantly modified by the unavailability of Cu in the

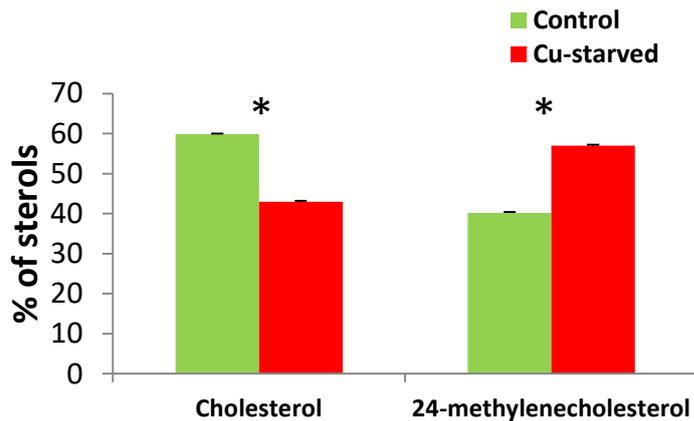
312 culture media, with a decreased cholesterol, while the 24-methylenecholesterol was increased

313 (Figure 3).



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 315 *Figure 2: Composition of A-B) polar (membranes) fatty acids (FA), C-D) neutral (storage) FA of P. delicatissima in the*  
 316 *control (green) and in the Cu-starved conditions (red). Zooms of the significant differences of FA in small proportions are*  
 317 *given in B) and D). Results are expressed as mean ± standard deviation. Statistical differences between the two conditions*  
 318 *are indicated as followed “NS” not significant, “\*” 0.01 < p-value < 0.05, “\*\*” 0.001 < p-value < 0.01, “\*\*\*” p-value <*  
 319 *0.001.*

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## 327 Discussion

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

*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<sub>PSII</sub>) 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<sub>PSII</sub>) provides a better picture of photosynthesis. The inhibition of rETR<sub>PSII</sub> 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

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

362 *Rearrangement of photosynthetic membranes.* Cu starvation induced a bottleneck in  
363 the photosynthetic electron flow that increases the excitation in the pigment antennae and  
364 favors the production of ROS and photodamages. However, under the light conditions of this  
365 experiment, the maximum PSII QY of *P. delicatissima* was only slightly decreased,  
366 highlighting some adaptation of the photosynthetic apparatus. Among the mechanisms to cope  
367 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 reinhardtii*, 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  $\mu\text{mol photons m}^{-2}$ ).

391 *Cu-starvation modifies the overall lipid metabolism.* Under the light conditions used  
392 for culture growth (130  $\mu\text{mol photon m}^{-2}$ ), 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

418 metabolism mainly reflects a storage of energy under the form of the mono-unsaturated FA  
419 16:1n-7. Other modifications may also originate from the *de-novo* synthesis of FA to meet the  
420 demand of new membrane lipids, especially for photosynthetic membranes to adapt to Cu  
421 starvation, or to replace FA peroxidated by ROS coming from photooxidation [13].

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

### 433 **Conclusion**

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

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

458

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