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1 Title: The redox state of the plastoquinone (PQ) pool is connected to  
2 thylakoid lipid saturation in a marine diatom

3 Kuan Yu Cheong<sup>1,2</sup> (ORCID: 0000-0002-9118-5797), Juliette Jouhet<sup>3</sup>, Eric Maréchal<sup>3</sup> and Paul G.  
4 Falkowski<sup>1,2,4</sup>

5 <sup>1</sup>Environmental Biophysics and Molecular Ecology Program, Department of Marine and Coastal Sciences,  
6 Rutgers, The State University of New Jersey, New Brunswick, NJ 08901

7 <sup>2</sup>Department of Plant Biology, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901

8 <sup>3</sup>Laboratoire de Physiologie Cellulaire et Végétale, Unité Mixte Recherche 5168, Centre National  
9 Recherche Scientifique, Commissariat à l'Energie Atomique et aux Energies Alternatives, INRAE,  
10 Université Grenoble Alpes, Grenoble Cedex 9, France

11 <sup>4</sup>Department of Earth and Planetary Sciences, Rutgers, The State University of New Jersey, Piscataway,  
12 NJ 08854

13 Author of correspondence

14 Paul G. Falkowski; falko@marine.rutgers.edu

15 Abstract

16 The redox state of the plastoquinone (PQ) pool is a known sensor for retrograde signaling. In this  
17 paper, we asked, “does the redox state of the PQ pool modulate the saturation state of thylakoid  
18 lipids?” Data from fatty acid composition and mRNA transcript abundance analyses suggest a strong  
19 connection between these two phenomena in a model marine diatom. Fatty acid profiles of  
20 *Phaeodactylum tricornutum* exhibited specific changes when the redox state of the PQ pool was  
21 modulated by light and two chemical inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which  
22 leads to the oxidation of the PQ pool, or 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB),  
23 which leads to the reduction of PQ. Data from liquid chromatography with tandem mass spectrometry  
24 (LC-MS/MS) indicated a ca. 7-20% decrease in the saturation state of all four conserved thylakoid lipids in  
25 response to an oxidized PQ pool. The redox signals generated from an oxidized PQ pool in plastids also  
26 increased the mRNA transcript abundance of nuclear-encoded C16 fatty acid desaturases (FADs), with  
27 peak upregulation on a timescale of 6 to 12 h. These results strongly suggest the presence of a  
28 retrograde signaling pathway. The connection between the redox state of the PQ pool and thylakoid

29 lipid saturation suggests a heretofore unrecognized signaling pathway that couples photosynthetic  
30 electron transport and the physical state of thylakoid membrane lipids.

31 Key words (alphabetical): Diatom, Lipid saturation, PQ pool, Redox state, Retrograde signaling, Thylakoid  
32 membrane

### 33 Introduction

34 Light and temperature affect the flux of excitation energy and photosynthesis, but how a plant  
35 cell responds to these environmental signals is not well understood. In eukaryotic algae, more than 90%  
36 of the proteins in the plastid are encoded in the nucleus (Abdallah et al., 2000; Allen et al., 2011;  
37 Grzebyk et al., 2003; Martin et al., 2002; Rujan & Martin, 2001). As the plastidome possesses only  
38 minimal set of genes to support photosynthesis (Oudot-Le Secq et al., 2007; Valentin et al., 1992),  
39 eukaryotic photosynthetic cells evolved retrograde signaling mechanisms that facilitate environmental  
40 cues between nuclear gene expression and proteins in the plastid. In this paper, we examine a potential  
41 signaling pathway responsible for thylakoid lipid saturation in a model marine diatom.

42 The oxidation of plastoquinol (PQH<sub>2</sub>) at the Q<sub>0</sub> site of cytochrome b<sub>6</sub>f complex (cyt b<sub>6</sub>f) is the  
43 slowest step in linear photosynthetic electron transport (PET) (Haehnel, 1977). Hence, the redox state of  
44 the plastoquinone (PQ) pool can potentially generate a redox signal to reflect the fluctuation in PET  
45 induced by environmental factors. In response to changes in light intensity, the redox state of the PQ  
46 pool regulates the expression of nuclear-encoded genes responsible for light harvesting pigment protein  
47 complexes (Chen et al., 2004; Escoubas et al., 1995; Yang et al., 2001). Similarly, the expression of  
48 nuclear-encoded pigment protein complexes is affected by changes in temperature, which also has been  
49 associated with the redox state of the PQ pool (Denis P. Maxwell et al., 1994). Furthermore, the redox  
50 state of the PQ pool transcriptionally regulates the stoichiometry of photosystem II (PSII) and  
51 photosystem I (PSI) in cyanobacteria, green algae and terrestrial plants (Karpinski et al., 1997; Li &  
52 Sherman, 2000; D. P. Maxwell et al., 1995; Pfannschmidt et al., 1999). Are there other cellular processes  
53 regulated by the redox state of the PQ pool?

54 The lipid composition in the plastid is characterized by a high abundance of non-phosphate  
55 containing glycolipids including sulfoquinovosyldiacylglycerol (SQDG),  
56 monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), as well as a low  
57 abundance of phosphoglycerolipids, such as phosphatidylglycerol (PG), phosphatidylcholine (PC) and  
58 phosphatidylinositol (PI) (Block et al., 1983; Douce et al., 1987; Mendiola-Morgenthaler et al., 1985).

59 Among them, SQDG, MGDG, DGDG and PG make up the four conserved lipids in thylakoid membranes in  
60 all oxygenic photoautotrophs. The acyl tails of these lipids are enriched in long-chain, unsaturated fatty  
61 acids and their saturation state heavily influences the viscosity of the hydrophobic membrane core  
62 (Harwood & Jones, 1989; Sarcina et al., 2003). For instance, an increase in thylakoid fatty acid saturation  
63 inhibits the rate of linear photosynthetic electron transport (PET) (Horváth et al., 1986; Öquist, 1982;  
64 Vigh et al., 1985). Decreasing fatty acid saturation is also connected to an increase in diffusion of  
65 quinone, a PQ-like lipophilic mobile electron carrier in mitochondrial respiration (Budin et al., 2018).  
66 Furthermore, thylakoid membranes are devoid of sterols (Nakamura & Li-Beisson, 2016). Without  
67 sterols to promote membrane cohesion, these membranes are especially susceptible to environmental  
68 conditions that alter their physical states (Beck et al., 2007; Dufourc, 2008a, 2008b). Therefore, a robust  
69 and rapid retrograde signaling mechanism is crucial to maintain an optimal biophysical state of thylakoid  
70 membranes for photosynthesis. However, little is known about the initial signals that trigger the  
71 modulation of the saturation state of thylakoid lipids. This knowledge gap is especially wide in diatoms,  
72 a group of unicellular eukaryotic photoautotrophs that often dominate in polar and temperate seas. In  
73 this paper, we tested the hypothesis that diatoms could alter the saturation state of their thylakoid  
74 lipids through a signal generated from the redox state of the PQ pool.

75 We present evidence that the redox state of the PQ pool is connected to thylakoid fatty acid  
76 saturation. Data from GC-MS analyses revealed that the fatty acid profiles, especially C16 fatty acids,  
77 exhibited specific changes in abundance when the PQ pool redox state was modulated independently by  
78 light or chemical inhibitors. Further examination with an oxidized PQ pool revealed: 1) decreases in the  
79 saturation state of all conserved thylakoid lipids and 2) increases in the mRNA transcript abundance of  
80 nuclear-encoded C16 fatty acid desaturase (FADs). These findings strongly suggest the presence of a  
81 retrograde signaling pathway that regulates the fluidity of thylakoid membranes.

## 82 Materials and methods

### 83 Culture maintenance, cell count and treatment

84 *Phaeodactylum tricornutum* Bohlin (CCAP 1055/1; strain Pt1 8.6) was maintained axenically in  
85 batch in artificial seawater enriched to F/2 levels at 18°C (R. R. Guillard, 1975; R. R. Guillard & Ryther,  
86 1962). Cells were grown under a 14/10 h light/dark cycle with white light emitting diodes at 500  $\mu\text{mol}$   
87 photons  $\text{m}^{-2} \text{s}^{-1}$  and 20  $\mu\text{mol}$  photons  $\text{m}^{-2} \text{s}^{-1}$  for high-light and for low-light acclimated cells,  
88 respectively. Cells used in all experiments were diluted to maintain log growth phase for at least three

89 consecutive generations (R. R. L. Guillard, 1973; Sunda et al., 2005). Cell densities were quantified using  
90 a Beckman Coulter Multisizer 3 (Beckman Coulter).

91 To oxidize the PQ pool of the cells, high-light acclimated cells were either shifted to the low light  
92 condition or incubated with sublethal concentrations of 3-(3,4-dichlorophenyl)-1,1-dimethylurea  
93 (DCMU; dissolved in absolute ethanol; Tokyo Chemical Industry) for 24 h. Conversely, low-light  
94 acclimated cells were shifted to the high light condition or incubated with sublethal concentrations of  
95 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone [DBMIB; dissolved in dimethyl sulfoxide (DMSO);  
96 Sigma-Aldrich] for equal amount of time to reduce the PQ pool (Durnford et al., 1998). We defined  
97 “sublethal concentrations” as the doses of DCMU and DBMIB that decreased growth rate, but still  
98 allowed photochemical activity (Figure 3). Doses which resulted in negative growth rate were excluded  
99 from analysis to prevent interference from the toxic effects. If not otherwise indicated, 25 nM of DCMU  
100 and 1.2 DBMIB were used in this study. All experiments were conducted at around 6 h after first light to  
101 minimize interference from circadian rhythm and photoperiod.

#### 102 Fatty acid composition analysis

103 For total cellular fatty acid analysis [i.e., fatty acids methyl esters (FAMES)], cells were harvested  
104 by filtration with glass fiber filters (GF/F; Whatman), followed by lipid extraction and transesterification  
105 (Bligh & Dyer, 1959; Rodríguez-Ruiz et al., 1998). The FAMES were analyzed using a Shimadzu GC-MS-  
106 QP2010S Gas Chromatography Mass Spectrometer (GC-MS; Shimadzu Scientific Instruments) equipped  
107 with a TRACE™ TR- FAME GC Column (60 m × 0.25 mm, film thickness 0.25 µm; Thermo Fisher Scientific),  
108 FID detector and quadrupole mass spectrometer. All detectable FAME species were identified by  
109 comparing the mass spectra to the National Institute of Standards and Technology 11 library. The  
110 relative abundance of each FAME species was corrected for extraction efficiency (Jenke & Odufu, 2012),  
111 quantified by referencing to a known amount of heptadecanoic acid internal standard (Sigma- Aldrich),  
112 and expressed as molecular percentage of total fatty acid. The double bond index (DBI) was determined  
113 with all detectable fatty acid species as described (Feijão et al., 2017).

114 We used liquid chromatography with tandem mass spectrometry (LC-MS/MS) to quantify fatty  
115 acids by lipid headgroups on the LIPANG platform, Grenoble, France, as described (Jouhet et al., 2017).  
116 Briefly, lipid extracts corresponding to 25 nmol of total fatty acids were dissolved in 100 µL of  
117 chloroform/methanol [2:1, (v/v); containing 125 pmol of each internal standard]. The internal standards  
118 were phosphatidylethanolamine (PE) 18:0-18:0 (Avanti Polar Lipid), diacylglycerol (DAG) 18:0-22:6  
119 (Avanti Polar Lipid) and SQDG 16:0-18:0 extracted from spinach thylakoids (Demé et al., 2014) and

120 hydrogenated (Buseman et al., 2006). The lipid extract was separated in lipid class by HPLC (Rainteau et  
121 al., 2012) and quantified by MS/MS. The HPLC separation was performed at 40°C using an Agilent 1200  
122 HPLC system equipped with a 150 mm × 3 mm (length × internal diameter) 5 µm diol column (Macherey-  
123 Nagel). The distinct glycerophospholipid classes were eluted successively as a function of the polar head  
124 group. The mass spectrometric analysis was done on a 6460 triple quadrupole mass spectrometer  
125 (Agilent) equipped with a Jet stream electrospray ion source. This quantification was done by multiple  
126 reaction monitoring (MRM) with 50 ms dwell time. Lipid amounts (pmol) were corrected for response  
127 differences between internal standards and endogenous lipids and by comparison with a quality control  
128 (QC). The QC extract, corresponding to a known *Phaeodactylum* lipid extract, was qualified and  
129 quantified by TLC and GC-FID (Jouhet et al., 2017).

130 Fast repetition rate (FRR) fluorescence measurement of PSII

131 DCMU irreversibly binds to the Q<sub>b</sub> site of PSII and blocks electron input into the PQ pool i.e.,  
132 oxidizing the PQ pool (Fedtke, 1982; Trebst, 2007). The inhibitory effect of DCMU was assessed by the  
133 time constant of Q<sub>a</sub> reoxidation ( $\tau_{Qa}$ ), determined by a three-component analysis on the fluorescence  
134 relaxation kinetics after a single turnover flash, using a custom built, miniaturized Fluorescence  
135 Induction and Relaxation (mini-FIRe) instrument (Gorbunov & Falkowski, 2020; Kolber et al., 1998).

136 mRNA transcript abundance analysis

137 For quantitative reverse transcription PCR (RT-qPCR) analysis, total cellular RNA was extracted  
138 using TRIzol Reagent (Invitrogen). The extracted RNA was purified by using a RNeasy MinElute Cleanup  
139 Kit (Qiagen). RT-qPCR was performed using an Applied Biosystem High-Capacity cDNA Reverse  
140 Transcription Kit (Thermo Fisher Scientific) and Power SYBR Green Master Mix (Thermo Fisher Scientific).  
141 Primers for the qPCR reaction were designed using Primer Express Software v3.0.0 (Thermo Fisher  
142 Scientific) and listed in Table S1. The reactions were assessed using a QuantStudio 3 Real-Time PCR  
143 System. The ribosomal protein small subunit 30S (RPS) gene was used as an internal standard (Siaut et  
144 al., 2007). The relative mRNA expression ratio (R) of C16 fatty acid desaturases (FADs) was expressed  
145 according to (Pfaffl, 2001).

146 Pigment composition analysis

147 Cells were harvested by filtration with GF/F filters (Whatman). The samples were wrapped in foil  
148 and flash frozen at -80 °C in liquid nitrogen. Pigment extraction and high-performance liquid  
149 chromatography (HPLC) analysis were performed as described (Wright et al., 1991). Briefly, pigment  
150 composition was analyzed with an Agilent 1100/1200 series HPLC system equipped with a G1315C Diode

151 Array Detector (scanning wavelengths 275-800 nm) and Zorbax Eclipse Plus C18 column (4.6x250mm; 5  
152  $\mu\text{m}$ ). The following HPLC Grade phase eluents were used: Solvent A (80% Methanol, 20% aqueous  
153 Ammonium Acetate, pH 7.2); Solvent B (90% Acetonitrile, 10% Water); Solvent C (100% Ethyl Acetate).  
154 Peaks were quantified at 440 nm. Pigments were identified automatically using the Agilent software,  
155 based on retention time and spectral shape. All peak area quantification and pigment identification were  
156 manually verified.

## 157 Statistical analysis

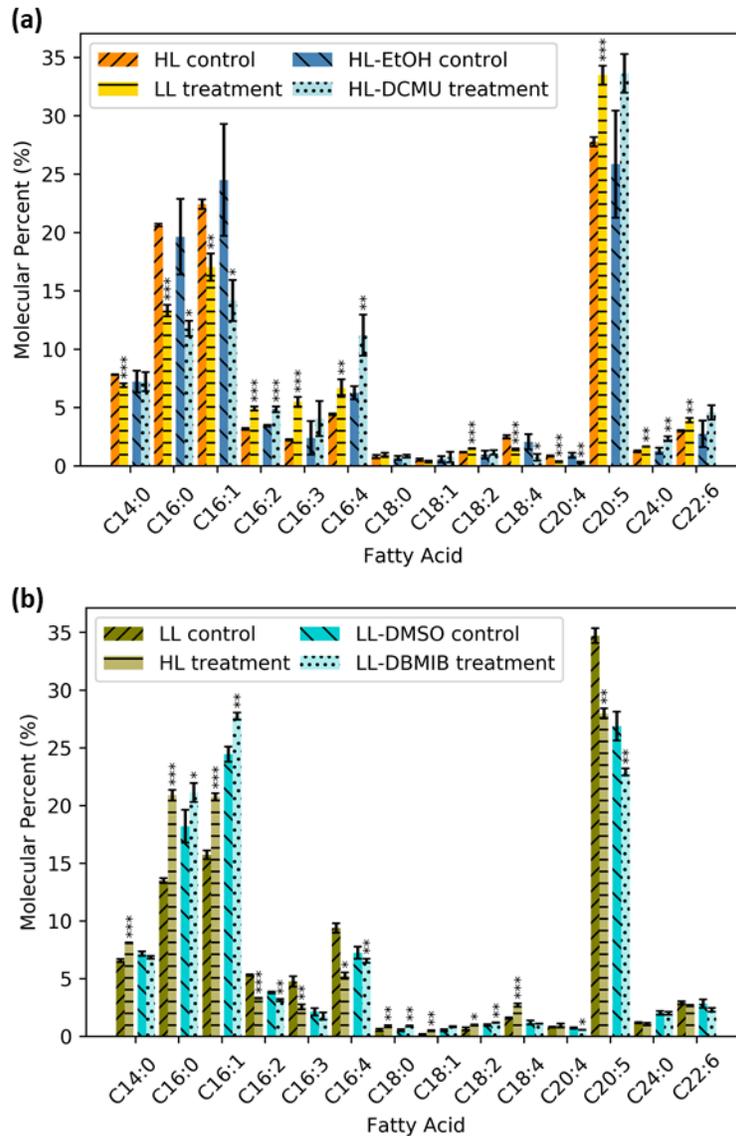
158 Normality was assumed in all analyses. Homoscedasticity of data was evaluated with the Levene  
159 test. If homoscedastic, an independent Student's t-test was performed (Section 3.1, 3.3-3.6;  
160 `scipy.stats.ttest_ind`, v1.6.2); otherwise, the Mann-Whitney U test was used (Section 3.3 and 3.5;  
161 `scipy.stats.mannwhitneyu`, v.1.6.2). One- way analysis of variance (ANOVA) was performed, followed by  
162 pairwise Tukey- HSD post-hoc test in Section 3.2 using a Python statistical package, Pingouin version  
163 0.33 (Vallat, 2018). All tests were performed at  $\alpha = 0.05$ .

164

## 165 Results

### 166 Total cellular fatty acid profiles

167 Fatty acid profiles exhibited specific changes depending on the redox state of the PQ pool (Fig.  
168 1). 12 out of the 14 fatty acid species (ca. 85%) responded identically, suggesting that an oxidized PQ  
169 pool modulates fatty acid abundance. When the PQ pool is reduced by high light or DBMIB, 11 out of 14  
170 (ca. 80%) fatty acid species responded in an identical manner. [Data](#) suggest that fatty acids respond in  
171 opposite directions depending on the redox state of the PQ pool (Fig. 2). Specifically, the FAs responsible  
172 for the changes were C16:0, C16:1, C16:2, C16:3, C16:4, C20:5, C24:0 and C22:6. Among these "redox  
173 sensitive" FAs, C16:0, C16:1, C16:2 and C16:4 exhibited significant and specific changes in response to  
174 the redox state of the PQ pool.



175

176 **Fig. 1. Cellular fatty acid profiles of *P. tricornutum* measured by gas chromatography-mass**  
 177 **spectrometry (GC-MS).** (a) Oxidation of the PQ pool with low light (LL) and 3-(3,4-dichlorophenyl)-1,1-  
 178 dimethylurea (DCMU) treatments for 24 h (b) Reduction of the PQ pool with high light (HL) and 2,5-  
 179 dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) treatments for 24 h. Values reported are  
 180 means  $\pm$  standard deviation (n = 3 biological replicates; except for HL treatment where n = 2). “\*”  
 181 represents  $p \leq 0.05$ , “\*\*” represents  $p \leq 0.01$  and “\*\*\*” represents  $p \leq 0.001$  ( $\alpha = 0.05$  with  
 182 independent t-test).

Fatty acid species	Direction of change relative to control			
	Oxidized PQ pool		Reduced PQ pool	
	<i>LL</i>	<i>DCMU</i>	<i>HL</i>	<i>DBMIB</i>
<i>C14:0</i>	Down***	Unchanged	Up***	Down
<b><i>C16:0</i></b>	<b>Down***</b>	<b>Down*</b>	<b>Up***</b>	<b>Up*</b>
<b><i>C16:1</i></b>	<b>Down**</b>	<b>Down*</b>	<b>Up***</b>	<b>Up**</b>
<b><i>C16:2</i></b>	<b>Up***</b>	<b>Up***</b>	<b>Down***</b>	<b>Down**</b>
<i>C16:3</i>	Up***	Up	Down**	Down
<b><i>C16:4</i></b>	<b>Up**</b>	<b>Up**</b>	<b>Down*</b>	<b>Down**</b>
<i>C18:0</i>	Up	Up	Up**	Up**
<i>C18:1</i>	Down	Up	Up**	Up
<i>C18:2</i>	Up***	Up	Up*	Up**
<i>C18:4</i>	Down***	Down*	Up***	Unchanged
<i>C20:4</i>	Down***	Down**	Unchanged	Down*
<i>C20:5</i>	Up***	Up	Down**	Down**
<i>C24:0</i>	Up**	Up**	Down	Down
<i>C22:6</i>	Up**	Up	Down	Down

183

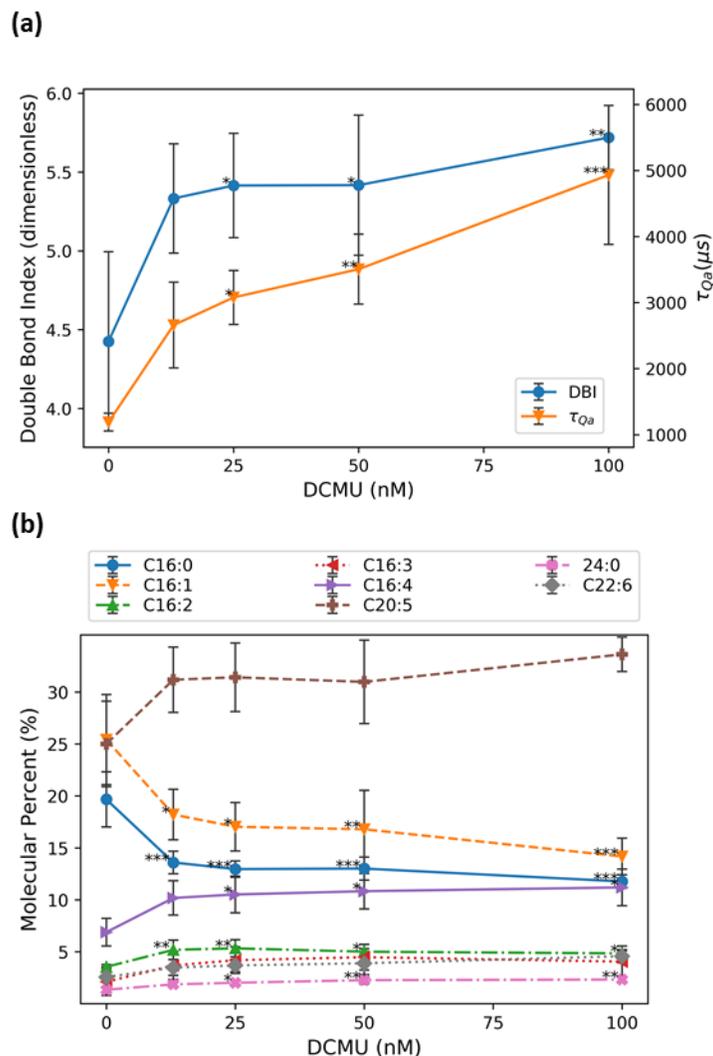
184 **Fig. 2. Changes in fatty acid abundance in response to the redox state of the PQ pool.** The direction of  
 185 change reported after 24 h of treatments are derived from values reported in Fig. 1. Fatty acid species  
 186 with significant opposite directions of change in oxidized versus reduced PQ pool are in bold. “\*”  
 187 represents  $p \leq 0.05$ , “\*\*” represents  $p \leq 0.01$  and “\*\*\*” represents  $p \leq 0.001$  at  $\alpha = 0.05$  with  
 188 independent t-test (n = 3 biological replicates; except for HL treatment where n = 2 biological replicates).

189

190 The saturation state of cellular fatty acids

191 We hypothesized that an oxidized PQ pool would lead to a decrease in the fatty acid saturation.  
 192 Indeed, increased concentrations of DCMU led to increased DBIs (Fig. 3a). Relative to the control, DBI  
 193 increased by 20.5%, 22.4%, 22.4% and 29.2%, as  $\tau_{Qa}$  increased by 125.1%, 159.0%, 194.0% and 312.0%.  
 194 Among the “redox sensitive” fatty acids, the abundance of C16:0 and C16:1 significantly decreased as  
 195 the concentration of DCMU increased (Fig. 3b). On the other hand, the abundance of less saturated  
 196 C16:2 and C16:4, and longer C24:0 significantly increased.

197

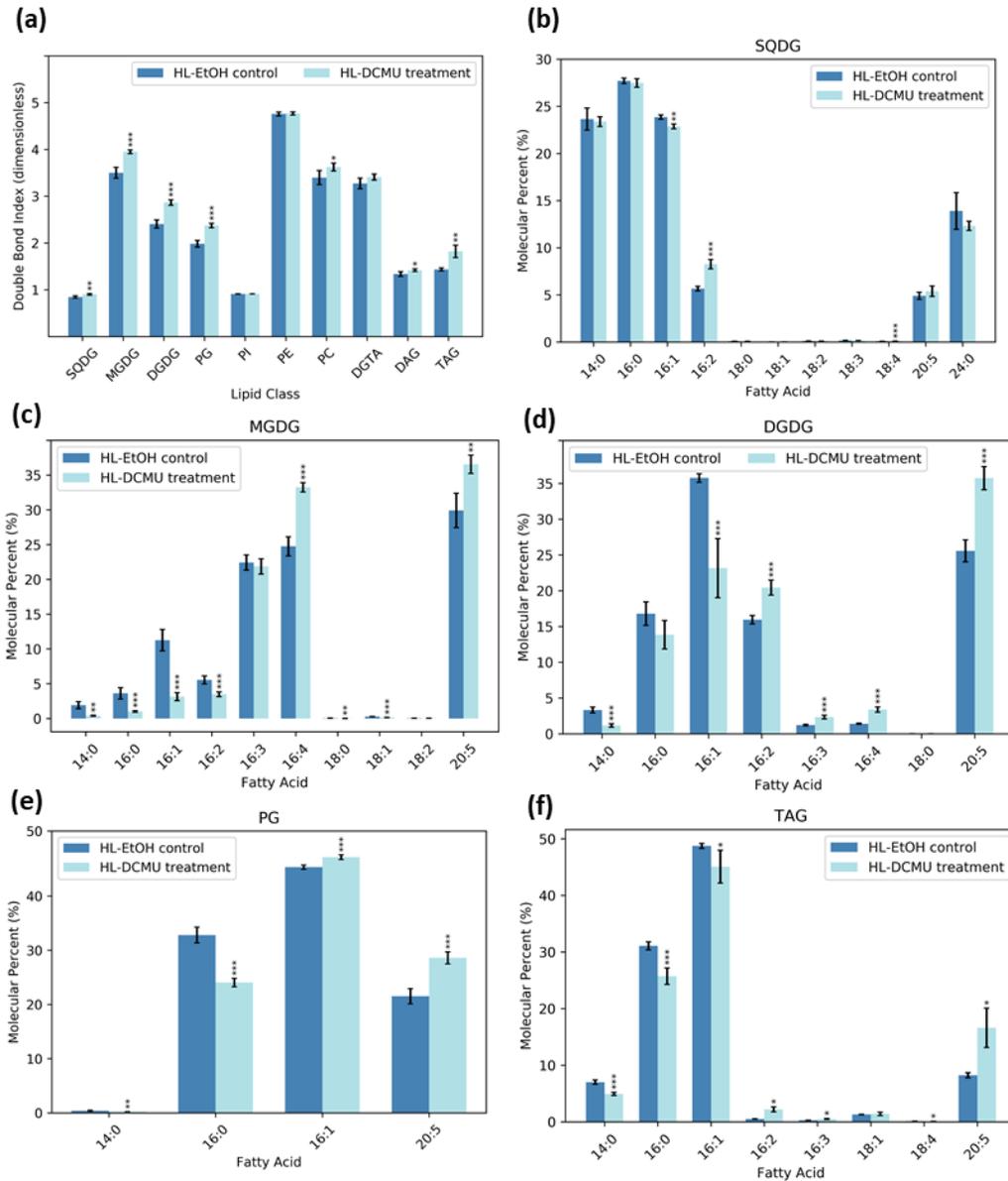


198  
 199 **Fig. 3. Fatty acid saturation state and abundance as a function of DCMU concentration.** (a) The double  
 200 bond index (DBI) and time constant of Qa reoxidation ( $\tau_{Qa}$ ), as a function of 3-(3,4-dichlorophenyl)-1,1-  
 201 dimethylurea (DCMU) concentration. DBI was computed from all detectable fatty acid species. (b) Fatty  
 202 acid abundance as a function of DCMU. The “redox sensitive” fatty acids identified in Fig. 2 are included  
 203 for brevity. Values reported are means  $\pm$  standard deviation ( $n = 3$  biological replicates). “\*” represents  $p$   
 204  $\leq 0.05$ , “\*\*” represents  $p \leq 0.01$  and “\*\*\*” represents  $p \leq 0.001$  ( $\alpha = 0.05$  with one-way ANOVA and  
 205 Tukey-HSD post-hoc test).

206

207 The saturation state of thylakoid lipids

208 The saturation state of thylakoid glycerolipids i.e., SQDG, MGDG, DGDG and PG, increased when  
209 the PQ pool was kept oxidized by DCMU (Fig. 4a). The DBI of non-thylakoid lipids such as PC, DAG and  
210 TAG were also affected, with their DBIs significantly increased by 6.7%, 6.1% and 27.0%, respectively.  
211 However, this increase in DBI seems to be specific. The DBI of PI, PE and DGTA were unchanged by an  
212 oxidized PQ pool. C16 fatty acids and C20:5 appear to contribute to most of the observed changes.  
213 C16:2 (45.9%) appeared to be the main contributor of the increase in SQDG (Fig. 4b). The largest  
214 significant changes observed in MGDG were C16:1 (-72.1%), C16:4 (34.3%) and C20:5 (22.2%; Fig. 4c).  
215 For DGDG, they were C16:1 (-35.3%), C16:2 (28.1%) and C20:5 (39.8%; Fig. 4d). Increase in DBI of PG  
216 could be mostly attributed to C16:0 (-26.7%) and C20:5 (32.9%; Fig. 4e). In TAG, C16:0 (-17.2%) and  
217 C20:5 (101.9%) led to the increase in the DBI.



218

219 **Fig. 4. The double bond index (DBI) of *P. tricornutum* measured by liquid chromatography with**  
 220 **tandem mass spectrometry (LC-MS/MS) (a) DBI of all detectable lipid species in diatoms treated with**  
 221 **ethanol (EtOH; carrier control) and 25 nM of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) for 24 h**  
 222 **are reported (b) DBI of (b) SQDG (c) MGDG (d) DGDG (e) PG (f) TAG fatty acid tails. Values reported are**  
 223 **means ± standard deviation (n = 4 biological replicates). “\*” represents p ≤ 0.05, “\*\*” represents p ≤**  
 224 **0.01 and “\*\*\*” represents p ≤ 0.001 (α = 0.05 with independent t-test for homoscedastic data and**  
 225 **Mann–Whitney U test for non-homoscedastic data). Abbreviations: SQDG,**  
 226 **sulfoquinovosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol;**  
 227 **PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC,**

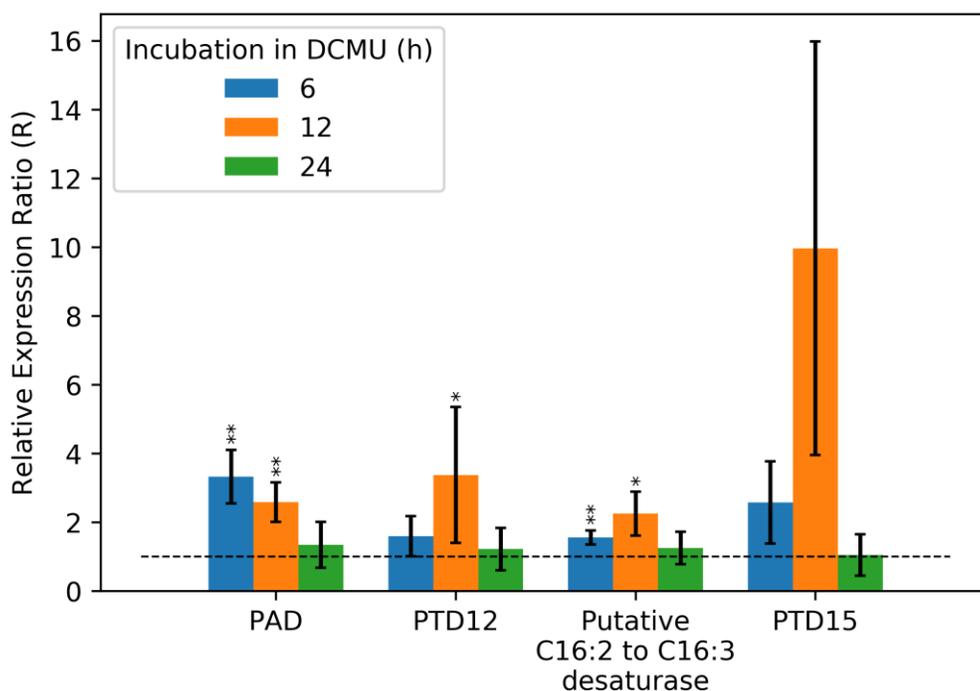
228 phosphatidylcholine; DGTA, diacylglycerol hydroxymethyltrimethyl- $\beta$ -alanine; DAG, diacylglycerol; TAG,  
229 triacylglycerol.

230

231 mRNA transcripts of C16 fatty acid desaturases

232 To examine if there is a connection between an oxidized PQ pool and nuclear-encoded FADs  
233 related to C16 fatty acids, we examined the transcription of the potential proteins. In *P. tricornutum*, the  
234 FAD PAD (palmitoyl-ACP desaturase; Phatr3\_J9316) is located on chromosome 1; PTD12  
235 (Phatr3\_J48423) on chromosome 17; Putative C16:2 to C16:3 desaturase (Phatr3\_EG02619) on  
236 chromosome 30; and PTD15 (Phatr3\_J41570) on chromosome 31. [Is the redox state of the PQ pool](#)  
237 [connected](#) to the transcription of these genes?

238 RT-qPCR measurements revealed that within 6 h of PQ pool oxidation there was a threefold  
239 increase in the transcription of the PAD, involved in the C16:0 to C16:1 desaturation, and a 1.6-fold  
240 increase in the putative C16:2 to C16:3 desaturase. At 12 h, most FADs were significantly upregulated,  
241 i.e., PAD by 2.6-fold; PTD12 by 3.4-fold and putative C16:2 to C16:3 desaturase by 2.3-fold. PTD15 was  
242 upregulated by almost 10-fold, despite the large standard deviation. It appears that this putative  
243 retrograde signaling pathway functions on a time scale of 6 to 12 h. 24 h of incubation did not  
244 [significantly](#) change the Rs of FADs tested.



245

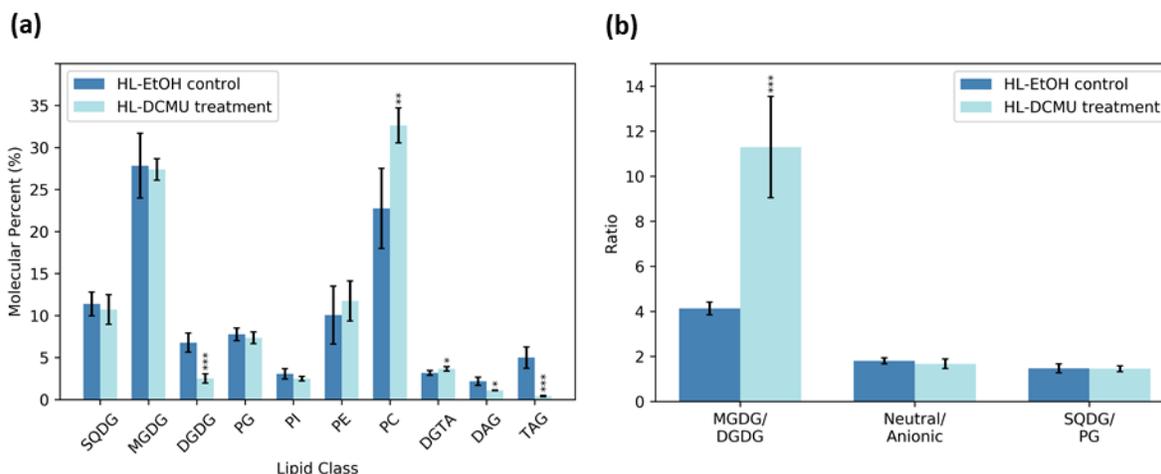
246 **Fig. 5. Relative mRNA expression ratio (R) of C16 fatty acid desaturases (FADs) after incubation in 25**  
247 **nM of DCMU for 6, 12 and 24 h.** R of a FAD is expressed in comparison to an internal standard ribosomal  
248 protein small subunit 30S (RPS) gene (Siaut et al., 2007), in DCMU treatments versus respective carrier  
249 controls (ethanol) according to (Pfaffl, 2001). We referred to Dolch & Maréchal (2015) and Smith et al.,  
250 (2021) for the function of each FAD. Dashed line at R of 1 indicates no change in expression level. Values  
251 reported are means  $\pm$  standard deviation (n = 3 biological replicates, except for PTD15 where n = 4  
252 biological replicates). “\*” represents  $p \leq 0.05$  and “\*\*\*” represents  $p \leq 0.01$  ( $\alpha = 0.05$  with  
253 independent t-test between target gene and RPS). Gene name (function) and Ensembl Protists ID: PAD  
254 (desaturates C16:0 to C16:1), Phatr3\_J9316; PTD12 (desaturates C16:1 to C16:2), Phatr3\_J48423;  
255 Putative C16:2 to C16:3 desaturase, Phatr3\_EG02619; PTD15 (desaturates C16:3 to C16:4),  
256 Phatr3\_J41570.

257

#### 258 Diatom lipid composition

259 In addition to the saturation state of thylakoid fatty acids, lipid composition can affect thylakoid  
260 structure and function by lipid phase behaviors. Thus, we examined if an oxidized PQ pool can modulate  
261 thylakoid lipid composition. Among the quartet of conserved thylakoid lipids, we observed significant  
262 changes only in DGDG (-63.0% relative to control; Fig. 6a) that led to an increased in MGDG/DGDG (Fig.  
263 6b). The charge distribution of thylakoid membranes i.e., neutral/anionic lipids, and composition of  
264 negatively charged lipids (SQDG and PG) appeared to be unchanged by the oxidation state of the PQ  
265 pool (Fig. 6b). Other lipids exhibiting significant changes include PC (43.4%), DGTA (15.5%), DAG (-49.8%)  
266 and TAG (-91.7%; Fig. 6a).

267



268  
 269 **Fig. 6. Lipid composition of *P. tricornutum* measured by liquid chromatography with tandem mass**  
 270 **spectrometry (LC-MS/MS)** (a) Sum of molecular abundance by lipid category. Diatom cells were treated  
 271 with ethanol (EtOH; carrier control) and 25 nM of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) for  
 272 24 h (b) The ratios of lipid classes. Neutral: MGDG and DGDG; Anionic: SQDG and PG. Values reported  
 273 are means  $\pm$  standard deviation ( $n = 4$  biological replicates). “\*” represents  $p \leq 0.05$ , “\*\*” represents  $p$   
 274  $\leq 0.01$  and “\*\*\*” represents  $p \leq 0.001$  ( $\alpha = 0.05$  with independent t-test with homoscedastic data;  
 275 Mann–Whitney U test for non-homoscedastic data). Abbreviations: SQDG,  
 276 sulfoquinovosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol;  
 277 PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC,  
 278 phosphatidylcholine; DGTA, diacylglyceryl hydroxymethyltrimethyl- $\beta$ -alanine; DAG, diacylglycerol; TAG,  
 279 triacylglycerol.

280

## 281 Discussion

282 Although the environmental cues (e.g., light and temperature) that trigger modulation of fatty  
 283 acid saturation are known, the initial sensing signal(s) remains elusive. It is well established that the  
 284 redox state of the PQ pool can generate retrograde signals to regulate photosynthetic pigments and  
 285 proteins on the order of minutes to days in cyanobacteria, green algae, diatoms and terrestrial green  
 286 plants (Escoubas et al., 1995; Karpinski et al., 1997; Lepetit et al., 2012; Li & Sherman, 2000; D. P.  
 287 Maxwell et al., 1995; Pfannschmidt et al., 1999). In this work, we examined the hypothesis that the  
 288 redox state of the PQ pool modulates the saturation state of thylakoid fatty acids. Our results strongly  
 289 suggest a connection between these two phenomena.

290 There was a *ca.* 85% agreement in changes of fatty acid abundance between LL and DCMU  
291 treatments i.e., an oxidized PQ pool. Reduction of the PQ pool by HL or DBMIB treatments resulted in a  
292 *ca.* 80% agreement in directions of abundance change (Fig. 1 and 2). DCMU treatments resulted in  
293 increased  $\tau_{Qa}$  (Fig. 3a) and, by inference, a more oxidized PQ pool. Thus, it appears oxidation of the PQ  
294 pool is connected to the increase in DBI. To definitively examining the role of the redox state of the PQ  
295 pool in thylakoid lipid modulation, future studies might include direct HPLC measurement of PQ, as  
296 demonstrated in (Khorobrykh et al., 2020). Nevertheless, biophysical analysis of the redox state of the  
297 PQ pool has been used for more than 40 years (Diner & Joliot, 1976). Our data suggest a strong  
298 correlation between total cellular DBI in thylakoid lipids and the redox state of the PQ pool.

299 Besides the four conserved thylakoid lipids, the DBI of DAG, TAG and PC also significantly  
300 increased. DAG serves as a precursor to membrane and storage lipids, and is thought to be present in  
301 the plastids and endoplasmic reticulum (ER) (Dolch & Maréchal, 2015). Although its exact localization in  
302 diatoms is not well known, PC is synthesized in the ER and thought to be the main extraplastidial lipid  
303 (Boudière et al., 2014; Dolch & Maréchal, 2015; Kroth et al., 2008). The storage lipid, TAG, exists in the  
304 form of droplets in *P. tricornutum* (Dolch & Maréchal, 2015). The increase of DBI in these non-thylakoid  
305 lipids and their importance to PET when PQ pool was oxidized is currently unclear. Nonetheless, the  
306 increase in DBIs appear to be specific as not all thylakoid lipids were affected indiscriminately. For  
307 instance, the DBI of PI, PE and DGTA were unchanged (Fig. 4a). Together, these data suggest a potential  
308 presence of a specific signaling mechanism in response to an oxidized PQ pool.

309 Many studies at a transcriptional level of regulation of FADs have been reported using light and  
310 dark treatments (Bai et al., 2016; Collados et al., 2006; Hernández et al., 2011; Kis et al., 1998; Nishiuchi  
311 et al., 1995). However, light and dark can affect a host of cellular processes. Therefore, it is challenging  
312 to pinpoint the sensing signal(s) that regulates FADs. DNA microarray analyses of DCMU- and DBMIB-  
313 treated cyanobacteria suggest that FADs are redox-responsive (Hihara et al., 2003). However, the  
314 changes in expression were similarly repressed and this might be attributed to the physiological artifacts  
315 from the higher concentration of inhibitors used (an order of magnitude higher than the present study).  
316 A recent study in diatoms pointed out that lower light intensity led to decrease in fatty acid saturation,  
317 and vice versa (Conceição et al., 2020). However, the signal(s) that led to this observation remain elusive  
318 and unexplored. Diatoms are secondary endosymbionts that possess four layers of plastid envelope  
319 membranes (Bowler et al., 2008, 2010). Retrograde signaling was once considered to be unlikely in  
320 diatoms because a signal from plastids must travel through these membranes to the nucleus (Wilhelm et

321 al., 2006). However, a focused analysis of the diatom plastid shows that it is bound to the outer nuclear  
322 envelope (oNE) by a direct connection with its outermost membrane, namely the chloroplast  
323 endoplasmic reticulum membrane (cERM)(Flori et al., 2016) In addition, at the level of the oNE-cERM  
324 isthmus, a membrane contact site links tightly the second outermost membrane of the plastid i.e., the  
325 periplastidial membrane (PPM) with the inner nuclear envelope (iNE) (Flori et al., 2016) The oNE-cERM  
326 isthmus and the iNE-PPM membrane contact site are therefore physical connections, which might  
327 determine highly efficient plastid-nuclear interactions in diatoms. Here, our data suggest that the  
328 change in fatty acid profile is connected to the redox state of the PQ pool transcriptionally (Fig. 5). An  
329 oxidized PQ pool led to an initial increase in R of PAD by 3.3-fold at 6 h, which desaturates C16:0 to  
330 C16:1 (Smith et al., 2021). This increase in C16:1 may serve as the substrate for downstream fatty acid  
331 desaturation, which is reflected by the peak upregulation of most C16 FADs at 12 h. Once the new lipid  
332 homeostasis is achieved at 24 h, all FADs exhibited unchanged expression relative to the internal  
333 standard gene. Together with the corresponding GC-MS and LC-MS/MS data, this expression pattern, in  
334 which FADs was upregulated to attain a new state of lipid homeostasis and downregulated once  
335 homeostasis was attained, suggests that the redox state of the PQ pool may function as a retrograde  
336 signal to regulate nuclear-encoded FADs.

337         The connection between the redox state of the PQ pool and saturation state of thylakoid fatty  
338 acids may have important physiological implications to the organism. Many crucial processes in  
339 photosynthesis, such as PET and PSII repair, rely heavily on diffusion (Goral et al., 2010; Kirchhoff, 2014;  
340 Kirchhoff et al., 2000). In PET, PQ and its reduced form, PQH<sub>2</sub>, are the only lipophilic mobile electron  
341 carriers that diffuse in the acyl tails of thylakoid lipids. Hence, decreased thylakoid fatty acid saturation  
342 induced by low light or DCMU (Fig. 1a, 2, 3a and 4a) is likely to decrease the viscosity of the  
343 microenvironment between PSII and cyt b<sub>6</sub>f. This may promote the rate of diffusion of PQ and PQH<sub>2</sub> to  
344 encourage higher electron input to PET. Conversely, saturated thylakoid membranes may retard the  
345 rates of PQ and PQH<sub>2</sub> diffusion to restrict electron input to PET in cells exposed to high light or DBMIB.  
346 We also explored the possibility if the redox state of PQ pool can act as a thermal sensor to modulate  
347 the saturation level of thylakoid fatty acids. According to the photostasis theory, low temperature and  
348 high light, both similarly exert high excitation pressure at PSII (Ensminger et al., 2006; Huner et al., 1998,  
349 2002; Wilson et al., 2006). Theoretically, DBMIB and low temperature treatments (inversely, DCMU and  
350 high temperature treatments) should result in similar fatty acid profiles. However, we did not observe  
351 these similarities in our cultures (data not shown). Thus, it is possible that additional sensors (or checks)  
352 are needed to trigger thermal modulation of thylakoid fatty acids. To the extent these experimental

353 results apply, the redox state of the PQ pool [may serve](#) as one of the signaling mechanisms to modulate  
354 FADs.

355 An oxidized PQ pool increases the abundance of light-harvesting complexes and the associated  
356 Chl  $\alpha$  (Escoubas et al., 1995; Maxwell et al., 1995). In accordance with literature, we observed *ca.* 70%  
357 increase in Chl  $\alpha$  in DCMU-treated cells (Fig. S1). Instead of light harvesting complex II in terrestrial  
358 plants, diatoms utilize fucoxanthin (Fx), chlorophyll a/c binding proteins (FCP) as the major peripheral  
359 light harvesting antennae for the photosystems. There are eight molecules of Chl  $\alpha$ , eight Fx and two Chl  
360  $c$  bound to each monomeric FCP (Premvardhan et al., 2010). Thus, a lack of corresponding increase in Fx  
361 and Chl  $c$  suggests that oxidation of the PQ pool alone did not significantly increase the abundance of  
362 FCPs, but mainly the [reaction centers](#). According to the percolation diffusion theory, high protein density  
363 encloses diffusion domain and prevents long-range diffusion of smaller hydrophobic molecules (Almeida  
364 & Vaz, 1995; Kirchhoff, 2014; Saxton, 1989). Thus, an increase in the abundance of [reaction centers](#),  
365 with unchanged thylakoid lipid abundance, may increase thylakoid protein density and impede lateral  
366 diffusion of lipophilic molecules. Thus, decreasing the saturation level of thylakoid fatty acids may  
367 counter this increase in membrane viscosity, for maintenance of lateral diffusion in the hydrophobic  
368 membrane core.

369 An oxidized PQ pool also led to changes in cellular lipid composition. We observed a *ca.* 60%  
370 decrease in DGDG (Fig. 6a) and corresponding 1.74-fold increase in MGDG/DGDG (Fig. 6b). MGDG  
371 (conical) does not promote bilayer forming whereas DGDG (cylindrical and forms hydrogen bonds with  
372 polar heads of lipids in adjacent bilayers) does (Demé et al., 2014; Williams, 1998). MGDG tends to form  
373 hexagonal II (HII) phase i.e., inverted tubules where lipid head groups form the center of the tubule and  
374 fatty acids are pointing outward to the aqueous phase (Jouhet, 2013; Seddon, 1990; Shipley et al.,  
375 1973). Decreasing saturation in fatty acids also favors the formation of the HII phase. Together, it  
376 appears that thylakoid membranes may favor the HII phase but not the lamellar phase (bilayer-forming  
377 and crucial for the maintenance of ultrastructure) when the PQ pool is oxidized. Data from  $^{31}\text{P}$ -NMR  
378 experiments on spinach thylakoids suggest that non-bilayer phases may play an important role in  
379 structural dynamics of thylakoid membranes (Garab et al., 2017). HII phase is important for the activities  
380 of some enzymes, including de-epoxidation of violaxanthin (Latowski et al., 2004). However, the cells  
381 examined exhibited decreased abundance of Ddx and unchanged Dtx (Fig. S1). They did not appear to  
382 activate non-photochemical quenching. Thus, this complex structure-function relationship of thylakoid  
383 lipids remains enigmatic. In phosphorus limiting condition, deficiency in PC could be replaced by DGTA

384 (Abida et al., 2015). However, our cells were grown in nutrient replete condition and thus the link  
385 between the redox state of PQ pool to these lipids is unclear. Future study should focus on the roles of  
386 these lipids in photosynthesis, especially in PET. Although it may seem straightforward, changes in fatty  
387 acid saturation have profound structural implications to biophysical properties of thylakoid membranes.  
388 In an *Arabidopsis* mutant deficient in *fad5*, semicrystalline PSII arrays were observed (Tietz et al., 2015).  
389 Instead of impeding the diffusion of small lipophilic molecules, the authors reported an enhancement of  
390 mobility of PQ and xanthophylls due to altered supramolecular protein organization. In diatoms, cryo-ET  
391 studies revealed semicrystalline arrays of PSII core subunits (Levitan et al., 2019). It will be interesting to  
392 study the effects of the PQ redox state on the supramolecular organization of PSII and PET.

393 This work provides a reference for future studies on elucidating the molecular intermediates  
394 connecting the plastid signal to nuclear gene expression in this retrograde signaling pathway.

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### 401 Author contribution

402 All authors contributed to the study conception and design. Material preparation, data collection and  
403 analysis were performed by Kuan Yu Cheong and Juliette Jouhet. The first draft of the manuscript was  
404 written by Kuan Yu Cheong and all authors commented on previous versions of the manuscript. All  
405 authors read and approved the final manuscript.

### 406 Data availability

407 The data that support the findings of this study are available from the corresponding author upon  
408 reasonable request.

### 409 Code availability

410 Not applicable.

### 411 Declarations

#### 412 Conflict of interest

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

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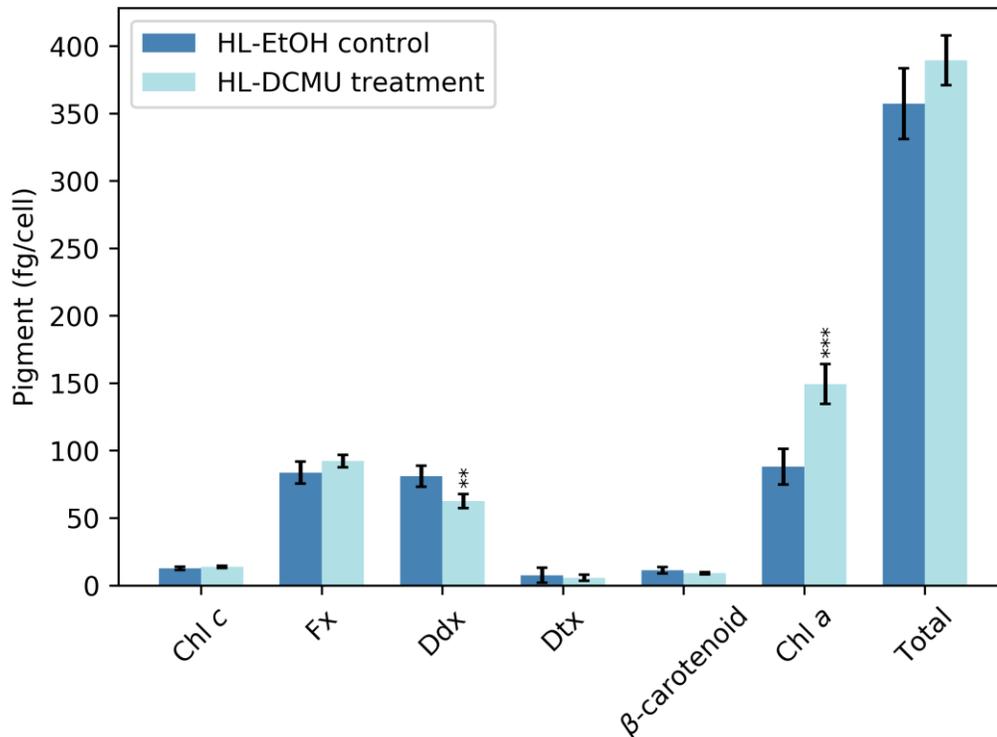
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664 **Supporting Information Fig. S1. Pigment composition of *P. tricornutum* incubated with 25 nM of 3-**  
 665 **(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) for 24 h.** Data collected by high-performance liquid  
 666 chromatography (HPLC). Values reported are means  $\pm$  standard deviation (n = 4 biological replicates).  
 667 “\*” represents  $p \leq 0.05$ , “\*\*” represents  $p \leq 0.01$  and “\*\*\*\*” represents  $p \leq 0.001$  ( $\alpha = 0.05$  with  
 668 independent t-test). Abbreviation: Chl c, chlorophyll c; Fx, fucoxanthin; Ddx, diadinoxanthin; Dtx,  
 669 Diatoxanthin; Chl a, chlorophyll a.

670 **Supporting Information Table S1. Primer sequences used for RT-qPCR.**

Gene	Ensembl Protists ID	Strand	Sequence (5'-3')
RPS (ribosomal protein small subunit 30S)	Phatr3_J10847	Forward	CGAAGTCAACCAGGAAACCAA
RPS (ribosomal protein small subunit 30S)	Phatr3_J10847	Reverse	GTGCAAGAGACCGGACATACC
PAD	Phatr3_J9316	Forward	TGCGGTGCTTGCCTTTC
PAD	Phatr3_J9316	Reverse	ACCCCGCAGATGTCTTCGTA

PTD12	Phatr3_J48423	Forward	CGATAAGGAGGGCGAGAAGA
PTD12	Phatr3_J48423	Reverse	CCGGTCGCACCGATCA
Putative C16:2 to C16:3 desaturase	Phatr3_EG02619	Forward	CTCCCGCGATCTTTTGGAG
Putative C16:2 to C16:3 desaturase	Phatr3_EG02619	Reverse	TGACCCAATCTGGCTGATAAGTT
PTD15	Phatr3_J41570	Forward	AGTGACGTTGAATGCCAAAAGA
PTD15	Phatr3_J41570	Reverse	ATCCCGTCGAGGATGGATT

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