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1 **Identification of circadian rhythms in *Nannochloropsis* species using bioluminescence**
2 **reporter lines**
3
4 Eric Poliner[1], Cameron Cummings[2‡], Linsey Newton[2], Eva M. Farré[1,2*]
5
6 [1] Cell and Molecular Biology Program, Michigan State University, [2] Department of Plant
7 Biology, Michigan State University, East Lansing, MI 48824, USA
8
9 Eric Poliner: ericpoli@msu.edu
10 Cameron Cummings: camerontcummings@gmail.com
11 Linsey Newton: newtonl2@msu.edu
12 Eva M. Farré: farre@msu.edu
13
14 [‡]Present address: Adimab, Lebanon, NH
15
16 Running head: Circadian rhythms in Nannochloropsis
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18 ***Corresponding author information:**
19 Eva M Farré
20 Department of Plant Biology
21 Michigan State University,
22 612 Wilson Road, Room 166
23 East Lansing, MI, 48824
24 Email: farre@msu.edu
25 Tel: 517-353-5215
26
27
28
29
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32 **Summary**
33 Circadian clocks allow organisms to predict environmental changes caused by the rotation of the
34 Earth. Although circadian rhythms are widespread among different taxa, the core components of
35 circadian oscillators are not conserved and differ between bacteria, plants, animals and fungi.
36 Stramenopiles are a large group of organisms in which circadian rhythms have been only poorly
37 characterized and no clock components have been identified. We have investigated cell division
38 and molecular rhythms in *Nannochloropsis* species. In the four strains tested, cell division
39 occurred principally during the night period under diel conditions, however, rhythms dampened
40 within 2-3 days after transfer to constant light. We developed firefly luciferase reporters for
41 long-term monitoring of *in vivo* transcriptional rhythms in two *Nannochlropsis* species, *N.*
42 *oceanica* CCMP1779 and *N. salina* CCMP537. The reporter lines express free-running
43 bioluminescence rhythms with periods of ~21-31 h that dampen within ~3-4 days under constant
44 light. Using different entrainment regimes, we demonstrate that these rhythms are regulated by a
45 circadian-type oscillator. In addition, the phase of free-running luminescence rhythms can be
46 modulated pharmacologically using a CK1 ε/δ inhibitor, suggesting a role of this kinase in the
47 *Nannochloropsis* clock. Together with the molecular and genomic tools available for
48 *Nannochloropsis* species, these reporter lines represent an excellent system for future studies on
49 the molecular mechanisms of stramenopile circadian oscillators.
50

51 **Significance statement**
52 Stramenopiles are a large and diverse line of eukaryotes in which circadian rhythms have been
53 only poorly characterized and no clock components have been identified. We have developed
54 bioluminescence reporter lines in *Nannochloropsis* species and provide evidence for the presence
55 of a circadian oscillator in stramenopiles; these lines will serve as tools for future studies to
56 uncover the molecular mechanisms of circadian oscillations in these species.
57

58 **Keywords**
59 circadian rhythms, stramenopiles, luciferase, transcription, cell division, bioluminescence
60 reporter, *Nannochloropsis oceanica*, *Nannochloropsis salina*, *Nannochloropsis* genus
61

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62 **Introduction**

63 Circadian clocks generate physiological rhythms of periods of ~ 24 h in the absence of external
64 cues. Circadian clocks are found in organisms that live exposed to daily light/dark cycles and
65 allow them to predict and adapt to changes in their environment. In photosynthetic organisms,
66 these clocks modulate photosynthetic capacity, growth, development, and responses to biotic and
67 abiotic stimuli and have been shown to be necessary for optimal growth and survival (Dodd *et*
68 *al.,* 2004, Gehan *et al.,* 2015, Ouyang *et al.,* 1998, Woelfle *et al.,* 2004, Yerushalmi *et al.,* 2011).
69 However, some cyanobacteria and plant species apparently lack self-sustained rhythms
70 (Gyllenstrand *et al.,* 2014, Holtzendorff *et al.,* 2008). Although evidence of rhythmic behavior
71 has been detected in a large number of species in different taxa, a good understanding of the
72 molecular components of circadian oscillators is only available for a few species of bacteria
73 (Cohen and Golden 2015), archeoplastida (green algae and plants)(Linde *et al.,* 2017, Noordally
74 and Millar 2015) and opisthokonts (animals and fungi) (Crane and Young 2014, Dunlap and
75 Loros 2017). Little is known about the molecular mechanisms underlying the circadian clock in
76 other eukaryotic lineages such as rhizaria, stramenopiles, or excavates.
77

78 In eukaryotes, circadian controlled transcription is regulated by a system of interlocked
79 transcription-translation feedback loops. These circadian oscillators can be reset or entrained by
80 environmental changes such as temperature and light, but are able to maintain similar periods
81 across a wide range of ambient temperatures, a characteristic known as temperature
82 compensation. In spite of the similarities among the structure of eukaryote circadian oscillators,
83 their key components are not conserved among taxa. Studies on one or two model organisms in
84 each taxon have driven our understanding of the mechanisms of these different circadian clocks
85 (Bell-Pedersen *et al.,* 2005). The clocks of green algae and plants are characterized by the
86 presence of pseudo-response regulators (PRRs) and the single MYB domain transcription factors
87 (LHY), which were initially identified and characterized in *Arabidopsis thaliana*. The search for
88 homologous genes have led the identification of clock components in other land plants and green
89 algae, such as *Ostreococcus taurii* (Corellou *et al.,* 2009, Linde *et al.,* 2017). In a similar
90 manner, the identification of the key components of animal clocks, which include basic helix-
91 loop-helix PAS (Per/Arnt/Sim) proteins such us CLOCK, CYCLE and BMAL and animal type
92 cryptochromes (Crane and Young 2014), was initially driven by work on fruit flies (Bargiello *et*

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93 *al.,* 1984, Zehring *et al.,* 1984). Comparative analyses then identified homologous clock genes in
94 mammals (Tei *et al.,* 1997). Work on the fungus *Neurospora crassa* led to the discovery of the
95 core components of fungal clocks, frequency (FRQ) and the LOV-domain containing light
96 receptor white collar 1 (WC-1) (Crane and Young 2014). In this case, conservation of clock
97 components has also been explored to investigate the role of the clock in other species (Hevia *et*
98 *al.,* 2015, Lee *et al.,* 2018).
99
100 Stramenopiles or heterokonts are a diverse group of secondary endosymbionts, in which a
101 previously non-photosynthetic eukaryote incorporated a red alga, and are phylogenetically
102 distant from the other photosynthetic lineages such as Viridiplantae (green algae and plants)
103 (Burki 2014). While stramenopiles include photosynthetic and non-photosynthetic groups, most
104 stramenopiles are aquatic and comprise one of the most abundant groups found in phytoplankton
105 (Rynearson and Palenik 2011). Transcriptome studies under light/dark cycles, have shown that a
106 large proportion of transcripts is diel regulated in stramenopiles (Ashworth *et al.,* 2013, Chauton
107 *et al.,* 2013, Gravot *et al.,* 2010, Poliner *et al.,* 2015, Smith *et al.,* 2016). Diel oscillations in
108 sporulation and gene expression have been also been observed in the oomycete *Phytophtora*
109 *infestans*, a non-photosynthetic stramenopile (Xiang and Judelson 2014). Self-sustained 24 h
110 rhythms under constant environmental conditions have been measured in a few stramenopiles
111 such as diatoms (Ragni and D'alcala 2007), brown algae (Schmid and Dring 1992, Schmid *et al.,*
112 1992) and *Nannochloropsis gaditana* (Braun *et al.,* 2014). However, other key characteristics of
113 circadian oscillators such as entrainment by environmental signals or temperature compensation
114 have not been investigated in stramenopiles.
115
116 Algae from the *Nannochloropsis* genus are a potential powerful model to characterize the
117 circadian clock in stramenopiles. This genus of small non-motile unicellular algae includes fresh
118 and marine species, although most studies have focused on salt water species due to their use as a
119 source of fish food and omega-3 fatty acids, and their biotechnological potential. Diel rhythms in
120 gene expression and cell division have been described in *N. oceanica* CCMP1779 and free
121 running 24 h rhythms in chlorophyll content have been reported in *N. gaditana* CCMP1779
122 (Braun *et al.,* 2014). Several *Nannochloropsis* strains from different worldwide locations have
123 been sequenced and current data suggests that *Nannochloropsis* species are haploid with genome

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124 sizes of ~30 Mb, containing from ~6,500 to ~12,000 genes (Corteggiani Carpinelli *et al.,* 2014,
125 Radakovits *et al.,* 2012, Schwartz *et al.,* 2018, Vieler *et al.,* 2012, Wang *et al.,* 2014). Recent
126 years have seen the development of a comprehensive set of molecular tools for working with
127 these algae, such as homologous gene replacement, overexpression, gene silencing and
128 CRISPR/Cas9 based genome editing (Kilian *et al.,* 2011, Poliner *et al.,* 2018a, Poliner *et al.,*
129 2018b, Poliner *et al.,* 2018c, Verruto *et al.,* 2018, Wang *et al.,* 2016, Wei *et al.,* 2017). In this
130 study, we describe the development of a bioluminescence reporter system for the long-term *in*
131 *vivo* measurement of gene expression in two *Nannochloropsis* species. We report a
132 comprehensive analysis of entrainment and light and temperature dependence of circadian
133 rhythms in *Nannochloropsis* genus, which form the base for future molecular characterization of
134 the circadian oscillator in this stramenopile.
135

136 **Results**

137 ***Nannochloropsis* species display oscillations in cell division under constant light conditions**

138 *N. oceanica* CCMP1779 cultures grown under 12 h/12 h light/dark cycles synchronize their cell
139 division, with DNA synthesis occurring during the second part of the light period and cytokinesis
140 occurring during the night (Poliner *et al.,* 2015). To test if cell division is regulated by a
141 circadian oscillator in *Nannochloropsis* species we measured cell numbers in cultures entrained
142 under light/dark cycles and released into constant light (Fig. 1a). We analyzed four lines from
143 two *Nannochloropsis* species originating from different latitudes (Figure S1). For our initial
144 experiments, we chose a lower light intensity for the free running condition (40 µmol m^{-2} s^{-1})
145 than the entrainment condition (100 µmol m^{-2} $s^{-}1$) as has been used for experiments in the green
146 marine algae *Ostreococcus tauri*. We observed cycling under light/dark for all four species.
147 However, although all lines tested displayed a gated cell division during the second day under
148 constant light conditions, two lines, CCMP537 and CCMP1778 did not display an oscillation
149 during the first day in constant light. This transient effect could be due to the differences in light
150 intensity under entrainment and free run conditions. We therefore analyzed growth in cells
151 entrained and released at the same light intensity of 40 µmol m^{-2} $s^{-}1$ (Fig. 1b). In this case, cell
152 division in both *N. salina* CCMP537 and *N. oceanica* CCMP1779 was gated during the first day
153 in constant light, but rhythms in the rate of cell division damped over the course of the
154 experiment (Fig. 1c). The period estimate for *N. salina* CCMP537 using FFT-NLLS was 31 h,

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155 but no period estimation was possible for *N. salina* CCMP1779. Similar results were obtained
156 with cells entrained and released at 100 µmol m^{-2} s^{-1} (Figure S2). These cell division rhythms in
157 *Nannochloropsis* species are weaker than the tight synchronization observed in green algae
158 (Goto and Johnson 1995, Moulager *et al.,* 2007) but similar to weak rhythms observed in the
159 diatom *Phaeodactylum tricornutum* (Ragni and D'alcala 2007).
160

161 **Development of luciferase reporter lines in *Nannochloropsis* species**

162 In order to carry out a more detailed analysis of the rhythms observed in *Nannochloropsis*
163 species, we developed bioluminescence reporter lines. We first tested three luciferase enzymes in
164 the line *N. oceanica* CCMP1779, the Firefly luciferase (FLUC), Renilla luciferase (RLUC) and
165 Nanoluciferase (NLUC). FLUC has been widely used as a circadian reporter in multiple systems
166 (Welsh *et al.,* 2005, Welsh and Kay 2005), however, RLUC and the engineered NLUC have the
167 advantage of being smaller proteins, and catalyze ATP-independent reactions (England *et al.,*
168 2016, Hall *et al.,* 2012). We have recently used NLUC to quantify protein expression in *N.*
169 *oceanica* CCMP1779 (Poliner *et al.,* 2018c). The codon optimized luciferase coding regions
170 were expressed under the control of the *CELLULOSE SYNTHASE* promoter (*CS*), a gene with
171 strong oscillations in RNA levels under light/dark cycles (Poliner *et al.,* 2015). All
172 *CS::LUCIFERASE* expressing lines displayed peaks of bioluminescence during the dark period
173 in accordance to the expression of the endogenous *CS* gene (Fig. 2a)(Poliner *et al.,* 2015).
174 Although RLUC and NLUC expressing lines showed high initial levels of luminescence both
175 reporters displayed a strong decrease of signal during the course of an experiment. In the case of
176 RLUC, this decrease is likely due to the chemical instability of its substrate, coelenterazine, in
177 aqueous solution (Andreu *et al.,* 2010). The NLUC substrate, furimazine, was initially selected
178 for its higher stability under animal tissue culture conditions (Hall *et al.,* 2012) and we tested
179 whether instability under our culture conditions could explain the decrease in signal. Refeeding
180 of substrate within a time course transiently increased the amplitude of oscillations of NLUC
181 expressing lines but had little effect on FLUC luminescence, indicating that the NLUC substrate
182 might be unstable under our experimental conditions (Figure S3). We also tested FLUC
183 expression under the control of the *light harvesting complex 8* gene (*LHC8*) promoter in *N.*
184 *oceanica* CCMP1779. The bioluminescence of this reporter peaked at dawn, which correlated to
185 the morning expression of the *LHC8* gene in this strain (Fig. 2a)(Poliner *et al.,* 2015).

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186
187 Under light/dark cycles, all the luciferase constructs tested led to oscillations in luciferase protein
188 content that were delayed with respect to the RNA level of the corresponding gene, *CS* or *LHC8*
189 (Fig. 2b, c). The doublet band in the *LHC8::FLUC* expressing lines is likely caused by an
190 alternative translational start site within the *LHC8* upstream sequence. The *in vivo* FLUC
191 bioluminescence correlated well with RNA levels of the *CS* gene (Fig. 2c). In contrast, the
192 maximum RLUC and NLUC *in vivo* bioluminescence were delayed with respect to the *CS* RNA
193 levels. These results indicate that *in vivo* firefly luciferase enzyme has a short catalytic activity
194 half-life in *Nannochloropsis* species. In animal cells, firefly luciferase also responds faster than
195 nanoluciferase (Hall *et al.,* 2012), which might be due to the product inhibition of the firefly
196 luciferase enzyme (Leitao and Esteves da Silva 2010). Therefore, FLUC *in vivo* luminescence in
197 the presence of substrate appears to reflect the rate of production of newly synthesized luciferase
198 enzyme, which in our case, correlates with its RNA content, in a similar manner to what has been
199 observed in higher plants (Farre and Kay 2007, Millar *et al.,* 1992).
200
201 Due to the robust activity of FLUC reporters in *N. oceanica* CCMP1779, we also developed
202 luciferase reporter lines in *N. salina* CCMP537. *N. salina* strains display oscillations in cell
203 division (Fig. 1) and the genome of *N. salina* CCMP537 has been sequenced (Wang *et al.,* 2014).
204 In *N. salina* CCMP537, the FLUC reporter under the control of its endogenous *CS* promoter also
205 maintained high amplitude oscillations under light/dark cycles with bioluminescence maxima
206 during the dark period (Fig. 3). As in *N. oceanica* CCMP1779, *LHC8* driven FLUC *in vivo*
207 bioluminescence in *N. salina* CCMP537 displayed a dawn peak of expression that weakened
208 during the course of the experiment. The differences in the time of maximum expression
209 between *CS* and *LHC8* driven FLUC in both *Nannochloropsis* species tested indicate that the
210 oscillations of *in vivo* FLUC activity are not due to changes of endogenous ATP levels, a
211 substrate of firefly luciferase, but reflect differences in transcriptional activity of these promoters
212 during the light/dark cycle. We therefore, focused on *N. oceanica* CCMP1779 and *N. salina*
213 CCMP537 *CS::FLUC* lines to further characterize the circadian rhythms of *Nannochloropsis*
214 species. For brevity, these lines are described from now on as *N. oceanica* and *N. salina* in the
215 text and figures.
216

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217 ***Nannochloropsis* species display damping oscillations in gene expression under constant**
218 **light conditions**
219 Under light/dark cycles, both *CS::FLUC* and *LHC8:FLUC* reporters displayed anticipatory
220 behavior under light/dark cycles indicating the presence of a endogenous oscillator (Fig. 2, 3).
221 To test if *Nannochloropsis* species maintain transcriptional rhythms under constant light we
222 measured *in vivo* bioluminescence in *N. salina* and *N. oceanica* expressing the *CS::FLUC*
223 reporter, which exhibited higher amplitude rhythms (Fig. 4). Since rhythms in the marine green
224 algae *Ostreococcus tauri* are lost at moderate to high light intensities we first tested
225 luminescence rhythms under different intensities of white light (Moulager *et al.,* 2007).
226 Bioluminescence traces were detrended and the periods estimated using Biodare 2 (Moore *et al.,*
227 2014)(Figure S4). After entrainment under light/dark cycles, *N. salina* maintained rhythms for 2-
228 3 cycles in constant white light conditions. The length of its free running period was ~27 h under
229 10 µmol m^{-2} s^{-1} and ~26 h under 40 µmol m^{-2} s^{-1}, but under 100 µmol m^{-2} s^{-1} of white light no
230 rhythmic cultures were observed. In contrast, *N. oceanica* displayed weaker rhythms than in *N.*
231 *salina* under constant white light, with fewer rhythmic cultures (Fig. 4). In particular, under 10
232 µmol m^{-2} s^{-1} white light, only 25% of the cultures were rhythmic based on our rhythmic criteria
233 (see Methods). Under weak red light, *N. salina* displayed erratic oscillations and no rhythmic
234 cultures, however, better rhythms were detected under 40 µmol m^{-2} s^{-1} red light with an average
235 period of 29 h. In contrast, *N. oceanica*'s rhythms were weaker under blue light than under red
236 light. In the dark under autotrophic conditions, firefly luciferase reporter activity in both
237 *Nannochloropsis* species was strongly decreased, as is the case with the marine unicellular algae
238 *Ostreococcus taurii* (Figure S5) (O'Neill *et al.,* 2011).
239
240 ***In vivo* luminescence rhythms are temperature compensated and are maintained under**
241 **short light/dark cycles**
242 One of the key characteristics of circadian oscillators is their ability to maintain similar circadian
243 periods under a wide range of temperatures, which is termed temperature compensation. We
244 investigated *CS::FLUC* driven luminescence under low white light and temperatures ranging
245 from 17°C to 28°C (Fig. 5a). *N. salina* displayed oscillations in all temperatures tested; all
246 cultures were rhythmic under 19°C and 22° C, but only ~60% were rhythmic at the higher
247 temperatures tested. In contrast, the rhythms were weak for *N. oceanica* at all temperatures tested

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248 and cyclic cultures were observed only under 19°C, 22°C and 25°C (Figure S6). In *N. salina* the
249 estimated period lengths showed great variability, ranging from 23 to 31 h (Fig. 5b). We
250 calculated the Q_{10}, the factor by which the rate of a reaction varies in response to a 10 °C change,
251 to quantify the degree of temperature compensation of *N. salina* bioluminescence rhythms. Using
252 all the available data we estimated the temperature compensation for transcriptional rhythms in
253 *N. salina* to have a Q_{10} of ~ 1.1, which lies within the range of other characterized circadian
254 oscillations in plants(Kusakina *et al.,* 2014) and algae (Anderson *et al.,* 1985)(Fig. 5b).
255
256 Environmental factors that entrain circadian oscillators (zeitgebers), such as light, can lead to
257 masking of the measured physiological rhythm. For example, light inhibits locomotor activity in
258 mice and therefore mice activity is arrhythmic under constant light conditions (Ohta *et al.,* 2005).
259 A similar effect could be involved in the damping rhythms of transcriptional luciferase reporters
260 under constant light conditions in *Nannochloropsis* species. Since circadian oscillators change
261 the time-dependent sensitivity towards the entrainment signal, in this case light, short light/dark
262 cycles can reveal free-running rhythms, a process called frequency-demultiplication, in
263 organisms in which masking occurs (Aschoff 1999). Therefore, when the endogenous period is a
264 multiple of the frequency of the light/dark cycle, organisms with a circadian oscillator maintain
265 their entrainment to the 24 h cycle and do not entrain to the new shorter cycles. To further test
266 whether a circadian oscillator is present in *Nannochloropsis* species, we measured rhythms under
267 short cycles of 3 h light and 3 h darkness (period = T= 6 h). Under T6 cycles both
268 *Nannochloropsis* species revealed no ultradian oscillations during the first day but small
269 amplitude 6 h oscillations were detectable the second day onwards, such that the *CS::FLUC*
270 reporter was induced in the dark and repressed in the light (Fig. 5c, Figure S7a). At 22° C *N.*
271 *oceanica* and *N. salina* cells maintained bioluminescence oscillations for three days with average
272 periods of 23.8 h and 28.6 h respectively (Fig. 5b, Figure S7c). As observed for the
273 bioluminescence rhythms under constant light, the circadian rhythms in *N. salina* were more
274 robust than the oscillations from *N. oceanica,* and 88% *N. salina CS::FLUC* cultures scored as
275 rhythmic in these experiments in contrast to 28% for *N. oceanica*. Similar period lengths were
276 observed in *N. salina* cells expressing the *LCH8::FLUC* reporter although the amplitude of these
277 oscillations was much reduced and the *LHC* reporters were sensitive to the short light/dark cycles
278 throughout the treatment (Figure S7b,c).

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279
280 We also quantified the circadian period at different constant temperatures in *N. salina* under T6
281 cycles (Fig. 5c,d). Rhythmic cultures were detected at all three temperatures tested (Fig. 5c, d).
282 Cultures at 19°C lacked ultradian oscillations during both the first and second day in T6
283 conditions indicating a more robust circadian regulation than at higher temperatures. *N. salina*
284 *CS::FLUC* rhythms under T6 had a Q_{10} of 1.2, similar to the rhythms under constant light
285 conditions (Fig. 5b,c).
286
287 **Luminescence rhythms in *Nannochloropsis* species respond to changes by light cues**
288 To determine if the *in vivo* luminescence rhythms could be reset by light we first carried out a
289 simulated jet lag experiment, in which *N. salina CS::FLUC* cells were entrained under light/dark
290 conditions and then treated with a night extension of 6 h (Fig. 6a). This treatment led to a phase
291 advance of 1-2 h in the first day after the shift; the *N. salina* cultures were able to entrain to the
292 new phase by the second day. The small phase shift and the delay in resetting indicates the
293 presence of an endogenous oscillator, such that the oscillations in luminescence are not only
294 driven by the light/dark cycles.
295
296 To further investigate light entrainment in *N. salina* we carried out phase shift experiments under
297 different light qualities. *N. salina CS::FLUC* cultures were grown under white 12h light/12 h
298 dark cycles, treated with dark periods of different durations and then transferred to constant
299 white, red or blue light conditions (Fig. 6b, c; Figure S8). As observed in the previous jet-lag
300 experiments an extension of the dark period led to a delay in phase that correlated with the length
301 of the dark extension (Fig. 6c,d, Figure S8). Dark periods shorter than 12 h led to phase advances
302 of 2-3 h, which were shorter than the observed delays (Fig. 6b,d, Figure S8). These delays and
303 advances were similar between blue and white light treatments; however, phase advances were
304 slightly stronger under red light. In the absence of a dark period, the phase was delayed under
305 blue and white light but not under red light. After the phase shifts, the new phase was maintained
306 during the second day under free running conditions indicating a change in the phase of a
307 circadian oscillator and not a direct light effect on the reporter expression (Fig. 6d). Under red
308 light conditions rhythms dampened quickly (Figure S8b), making it difficult to determine the

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309 phase of the second peak of luminescence. These experiments further indicate the presence of a
310 circadian oscillator in the genus *Nannochloropsis*.
311
312 Another characteristic of circadian oscillators is that they determine the phase relationship or
313 phase angle of gene expression within a 24 h light/dark cycle (T24). Circadian control leads to
314 phase advances when the cycles are longer than 24 h and two phase delays when the cycles are
315 shorter than 24 h (Johnson *et al.,* 2003). We determined the phase angle, the relative time of peak
316 expression of the *CS::FLUC* reporter within one cycle, in *N. salina* and *N. oceanica* under T20,
317 T22, T24 and T26. Both species displayed stable entrainment under these conditions (Fig. 6e and
318 f), however, the relative time of expression of the reporter within the light/dark cycles was
319 advanced under T26 and delayed under T20 and T22 (Fig. 6g and h) supporting the presence of
320 an endogenous oscillator.
321
322 **Entrainment of luminescence rhythms by temperature**
323 In most organisms, temperature, in addition to light, plays an important role in resetting the
324 circadian clock (Rensing and Ruoff 2002). We investigated entrainment of *CS::FLUC*
325 oscillations in *Nannochloropsis* species by growing cultures under temperature cycles of 12 h
326 27°C and 12 h 17°C under constant light conditions. Temperature cycles were able to maintain
327 robust high amplitude oscillations in both *N. salina* and *N. oceanica* (Fig. 7). The peak of
328 *CS::FLUC* expression in both species occurred at the end of the warm period, an earlier phase
329 than light entrainment. This phase relationship was maintained when cultures were transferred to
330 constant temperature and light conditions. As observed for light entrained cultures, only a few *N.*
331 *oceanica* cultures were rhythmic after temperature entrainment (Figure 7). For *N. salina*, the
332 estimated average period of the luminescence rhythm after temperature entrainment, 28.8 h, was
333 similar to the period after light entrainment.
334
335 **Potential clock components in the *Nannochloropsis* genomes**
336 No circadian clock component has been identified in stramenopiles, however, *Nannochloropsis*
337 genomes encode for genes with some similarities to plant and animal clock genes (Vieler *et al.,*
338 2012). Proteins containing plant-specific CCT domains are involved in circadian and
339 photoperiod control in land plants and green algae (Farre and Liu 2013, Serrano *et al.,* 2009). We

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340 identified three CCT containing proteins annotated in the *N. oceanica* and the *N. gaditana*
341 genomes and one in the *N. salina* genome (Figure S9). The CCT domains across stramenopiles
342 display higher similarity to each other than to CCT domains outside that group. The expression
343 of two of the CCT *N. oceanica* genes cycle under light/dark conditions (Figure S10a) (Poliner *et*
344 *al.,* 2015). None of the currently annotated stramenopile CCT domain proteins appear to contain
345 either response-regulator or BBOX domains, which are characteristic in green algae and land
346 plant proteins with circadian or photoperiod function, and therefore, their potential role in the
347 clock remains unclear.
348
349 *Nannochloropsis* species contain an animal cryptochrome like protein, homologe to the diatom
350 CPF1 (cryptochrome photolyase family) (Vieler *et al.,* 2012) (Figure S11). Diatom CPF1 has
351 been shown to be able to inhibit the transcriptional activity of the mouse clock proteins BMAL
352 and CLOCK in a heterologous system, and has been proposed to be involved in circadian
353 regulation (Coesel *et al.,* 2009). BMAL1 and CLOCK are part of the bHLH-PAS protein family,
354 whose members are involved in circadian regulation in animals (Crane and Young 2014). bHLH-
355 PAS proteins are not found in green algae or land plants but have been identified in red algae and
356 stramenopile genomes including *Nannochloropsis* species (Thiriet-Rupert *et al.,* 2016, Vieler *et*
357 *al.,* 2012)(Figure S12). Three of the four *N. oceanica* CCMP1779 bHLH-PAS proteins cycle
358 under light/dark conditions (Figure S10b). In stramenopiles, these proteins contain only one PAS
359 domain instead of the two PAS domains found in animal proteins (Figure S12a) and therefore it
360 has been suggested that the stramenopile proteins are an example of convergent evolution
361 (Thiriet-Rupert *et al.,* 2016).
362
363 Casein kinase 1 (CK1) is a conserved component of circadian systems in eukaryotes and the
364 pharmacological inhibition of CK1 affects the circadian period in mammals (Badura *et al.,* 2007,
365 Eide *et al.,* 2005, Walton *et al.,* 2009), *Drosophila melanogaster* (Meng *et al.,* 2010),
366 *Neurospora crassa* (Querfurth *et al.,* 2011), *Ostreococcus tauri* (van Ooijen *et al.,* 2013) and
367 *Caenorhabditis elegans* (Goya *et al.,* 2016). *N. oceanica* contains a CK1 gene with 72% identity
368 with mouse casein kinase 1 ε (NannoCCMP1779|10930); this gene cycles under light/dark
369 conditions with a peak at dawn (Figure S13a). To further test a possible involvement of a
370 circadian oscillator in *Nannochloropsis* species, we investigated the effect of the CK1 ε/δ

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371 inhibitor PF-670462 on bioluminescence oscillations. In *N. salina*, PF-67462 led to phase delays
372 of *CS::FLUC* expression in a concentration dependent manner (Fig. 8), although the free running
373 period was only reduced in two of the tested concentrations (Figure S13b). PF-670462 treatment
374 leads to a lengthening of the period in several organisms and causes phase delays in mammals
375 (Badura *et al.,* 2007, van Ooijen *et al.,* 2013, Walton *et al.,* 2009). These results suggest that
376 phosphorylation plays a key role in circadian control in *N. salina*.
377

378 **Discussion**

379 Circadian studies in stramenopiles have been limited to the measurement of physiological
380 outputs such as cell division, pigment content or photosynthesis rates that are difficult to apply
381 for high throughput studies (Makarov *et al.,* 1995, Ragni and D'alcala 2007). In this work, we
382 describe oscillations in cell division in several *Nannochloropsis* species and used a luciferase
383 reporter based system to characterize the circadian oscillations in the *Nannochloropsis* genus. In
384 spite of the damping of cell division and transcriptional rhythms under free running conditions,
385 the *Nannochloropsis* circadian clock displays key characteristics of a biological oscillator
386 (Roenneberg *et al.,* 2005). This oscillator can be entrained by light and temperature, causes jet
387 lag, leads to changes in the phase angle when entrained at periods slightly different than 24 h and
388 leads to frequency demultiplication when the entraining period is much shorter than 24 h.
389

390 Under light/dark cycles mitosis occurs during the dark period in *N. oceanica* (Poliner *et al.,*
391 2015)(Figure 1) and we observed a similar phase of cell division in the four *Nannochloropsis*
392 strains tested. Although under constant light conditions cell division rhythms were often weak,
393 cell division occurred during the subjective night period. Similar phases of cell division under
394 light/dark and constant light conditions have been observed in many unicellular and multicellular
395 eukaryotic algae (Anderson *et al.,* 1985, Goto and Johnson 1995, Johnson 2010, Makarov *et al.,*
396 1995, Moulager *et al.,* 2007). It has been proposed that this particular phase of cell division is
397 due to the increased UV sensitivity of the cells during the nuclear division process (Nikaido and
398 Johnson 2000). It has also been proposed that the temporal separation of cell division from
399 photosynthesis protects cells from excessive oxidative stress (Miyagishima *et al.,* 2014).
400

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401 Bioluminescence reporters have been used widely for analysis of circadian transcriptional
402 rhythms in a large number of species (Welsh *et al.,* 2005, Welsh and Kay 2005). We have tested
403 several luciferase reporter genes in two *Nannochloropsis* species and shown that a firefly
404 luciferase gene is able to track *in vivo* RNA oscillations and allows for long-term monitoring of
405 transcription. In contrast, the *in vivo* luminescence of the small synthetic nanoluciferase tracked
406 protein levels well but was unsuitable for long term *in vivo* monitoring. We used firefly
407 luciferase expressing lines to analyze the fundamental properties of circadian oscillators in
408 *Nannochloropsis*.
409
410 In contrast to the strong sustained rhythms in other unicellular algae (Noordally and Millar
411 2015), cell division and bioluminescence rhythms in *Nannochloropsis* species damped within 3-4
412 days under constant light conditions. The rhythms of *N. salina* CCMP537 appear slightly more
413 robust than the ones from *N. oceanica* CCMP1779. The period length estimations of the
414 bioluminescence rhythms had high variability, which might be caused by the rapid damping. In
415 *N. salina* these rhythms appeared to be temperature compensated and had average periods of
416 ~24-31 h. The damping of these rhythms was observed when using different promoters
417 indicating that the decrease in amplitude was not promoter specific, although it is possible that
418 other genes show more sustained oscillations. Future studies analyzing the genome wide
419 transcriptome oscillations will allow us to determine the extent of circadian regulation of RNA
420 levels in *Nannochloropsis* species. It is possible that constant light conditions inhibit
421 transcriptional rhythms in this genus. Constant light can lead to arrhythmicity in other organisms.
422 Circadian rhythms in the green algae *Ostreococcus tauri* are abolished under constant medium to
423 high light intensities (Moulager *et al.,* 2010), locomotor rhythms in animals and conidiation
424 rhythms in *Neurospora crassa* are absent under constant light conditions (Pittendrigh and Daan
425 1976, Schneider *et al.,* 2009), and dim light inhibits the rhythms of fruit flies (Winfree 1974).
426 However, in *Nannochloropsis* species, we observed a similar degree of damping under high
427 frequency T6 cycles (3 h light/3h dark) and under constant light. After one day in T6 the
428 luminescence reporters started to react to the imposed light/dark cycles and this loss of gating
429 correlated with the damping of the ~24 h rhythms in constant light (Figure 5), indicating that
430 masking is not the cause of the damping of free running rhythms.
431

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432 There are several potential explanations for the poor free running rhythmicity in
433 *Nannochloropsis* species. The observed damping might be caused by desynchronization of the
434 single cells in the culture as is the case of mammalian fibroblasts (Welsh *et al.,* 2004). It is also
435 possible that the experimental conditions, including media composition, are not optimal for
436 rhythmicity in the species studied (Doherty and Kay 2010, Hurley *et al.,* 2014, Olivares-Yanez *et*
437 *al.,* 2016). The transcriptional rhythms in *Nannochloropsis* species could also be controlled by a
438 damped oscillator. The dinoflagelate *Lingulodium*, a model of early circadian studies due to its
439 robust endogenous bioluminescece rhythms, does not appear to have strong RNA oscillations
440 under constant light conditions (Roy *et al.,* 2014) although it does display rhythms in protein
441 synthesis (Morse *et al.,* 1989, Morse *et al.,* 1990). Future studies will reveal the role of
442 posttranscriptional regulation of the circadian rhythms in the *Nannochloropsis* genus.
443
444 In organisms with robust circadian clocks, these clocks provide a fitness advantage (Dodd *et al.,*
445 2005, Woelfle *et al.,* 2004, Yerushalmi *et al.,* 2011). However, it remains unclear whether this
446 selective advantage requires a robust self-sustained circadian oscillator or whether weaker
447 oscillators can play a similar role. There are several photosynthetic organisms with no apparent
448 free running oscillations. Under constant light conditions no oscillations of gene expression and
449 chlorophyll fluorescence have been detected in the gymnosperm Norway Spruce (*Picea abies*) in
450 spite of containing the canonical plant clock genes (Gyllenstrand *et al.,* 2014). In contrast to the
451 widely studied *Synechococcus elongatus*, other cyanobacteria such as the abundant
452 *Prochlorococcus* genus lack a functional *kaiA* gene and no rhythms have been detected under
453 constant light conditions (Holtzendorff *et al.,* 2008, Schmelling *et al.,* 2017). It has been
454 hypothesized that an hour-glass oscillator that is reset every day is sufficient for these
455 *Prochlorococcus* species because they originate from southern latitudes where they do not
456 experience large changes in daylength, and their marine environment is more stable than fresh
457 water habitats (Mullineaux and Stanewsky 2009). A recent study shows that diel rhythms are
458 stronger in *Prochlorococcus* in co-culture with a heterotrophic bacterium suggesting that biotic
459 interactions can help maintain oscillations in plankton communities (Biller *et al.,* 2018).
460 Experiments using arrhythmic and weakly rhythmic mutants in *S. elongatus* indicate that lines
461 with a damped oscillator grow better than the wild type under light/dark cycles (Woelfle *et al.,*
462 2004) which suggests that a robust clock is not required for optimal growth under diel

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463 conditions. Non-motile phytoplankton are likely to be exposed to low light or dark conditions
464 due to water circulation and turbulence. A weak damped oscillator might be faster to reset than a
465 robust oscillator allowing for faster entrainment (Bordyugov *et al.,* 2015) and might be of
466 advantage in non-motile marine plankton.
467
468 *Nannochloropsis* bioluminescence rhythms can be entrained by light and temperature cycles. The
469 similarity between the blue and white light phase response curves indicate that a blue light
470 receptor might mediate the main light input signal to the clock. *N. salina* phase response curves
471 using blue light are similar to the ones observed in the green marine algae *Ostreococcus tauri*
472 under blue light, and both display stronger advances than delays (Thommen *et al.,* 2015). In
473 contrast to diatoms, *Nannochloropsis* species lack phytochromes (Vieler *et al.,* 2012) and it is
474 unclear whether they contain any red light photoreceptors. *N. oceanica* encodes for three
475 Aureochrome genes (Vieler *et al.,* 2012). Aureochromes are stramenopile specific
476 photoreceptors that have been shown to be involved in blue light mediated photomorphogenesis
477 in the *Vaucheria* genus and in the induction of cell division by blue light in diatoms (Kroth *et al.,*
478 2017, Takahashi *et al.,* 2007). Marine waters are enriched in blue light since light of longer wave
479 lengths cannot penetrate water efficiently (Kirk 2011, Ragni and Ribera D'Alcalà 2004) and
480 therefore blue light is likely to play a key role in signaling in marine organisms. Aureochromes
481 contain conserved bZIP DNA-binding and LOV light-sensing motifs (Takahashi *et al.,* 2007),
482 dimerize, and their DNA binding affinity is modulated by light exposure (Banerjee *et al.,* 2016,
483 Heintz and Schlichting 2016, Herman *et al.,* 2013, Hisatomi *et al.,* 2013). Future studies will
484 define the role of Aureochromes in the light input to the clock in stramenopiles.
485
486 No circadian clock components have been identified in the genus *Nannochloropsis*.
487 *Nannochloropsis* species appear to lack clear homologs to plant clock components, although
488 CCT domain containing proteins are interesting potential regulatory candidates. As other
489 stramenopiles, Nannochloropsis species also encode for several proteins similar to clock
490 components found in animals. *Nannochloropsis* CPF1 like proteins, as diatom CPF1, show
491 similarities to 6-4 photoylases in plants and animals as well as to cryptochromes proteins
492 involved in circadian regulation in insects and mammals (Figure S11). CPF1 from
493 *Phaeodactylum tricornutum* as well as CPF1 from the green alga *Ostreococcus tauri* are able to

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494 inhibit the transcriptional activity of the mouse BMAL-CLOCK complex in a heterologous
495 system, the same activity as the mouse circadian clock component CRY1 (Coesel *et al.,* 2009,
496 Heijde *et al.,* 2010). However, although *O. tauri* does not encode for bHLH-PAS proteins, the
497 presence of bHLH-PAS proteins in stramenopiles suggest that these interactions might be
498 functionally relevant in stramenopiles.
499

500 **Conclusion**

501 Using bioluminescence transcriptional reporters we provide evidence for the presence of
502 circadian rhythms in *Nannochloropsis* species. The *N. salina* luminescence strains characterized
503 in this study represent a powerful system for future studies to identify the mechanisms regulating
504 circadian rhythms in stramenopiles. In addition to the luminescence reporters described in this
505 study, the recent development of a comprehensive set of molecular tools for *Nannochloropsis*
506 species (Poliner *et al.,* 2018a) will allow the characterization of the circadian function of the
507 putative clock genes, the investigation of the role of photoreceptors in entrainment, and the
508 development of mutant screens to identify lines with compromised rhythms. Future studies on
509 the clock in stramenopiles will not only improve our understanding of the role of circadian
510 clocks in the biology of marine phytoplankton but also provide new insight into the evolution
511 and design principles of circadian oscillators.
512

513 **Experimental procedures**

514 ***Nannochloropsis* species and growth conditions**

515 The *Nannochloropsis* lines *N. oceanica* CCMP1779, *N. salina* CCMP537, *N. salina*
516 CCMP1778, and *N. salina* CCMP1776, were originally collected at different latitudes (29°N,
517 41.6°N, 55.75°N, and 55.75°N respectively) as described at Bigelow National Center for Marine
518 Algae and Microbiota (Fig. S1). We used the genome sequences and annotation versions
519 *Nannochloropsis oceanica* CCMP1779 v1.0 (Vieler *et al.,* 2012) available at JGI and
520 *Nannochloropsis salina* CCMP537 (Wang *et al.,* 2014) available at
521 http://www.bioenergychina.org:8989/index.html. Liquid cultures were grown in flasks in f/2
522 medium and maintained on a shaker set at 120 rpm. If not otherwise indicated cultures were
523 grown under white fluorescent lights. Blue and red light treatments were conducted using LED
524 lights; their spectrum is shown in Figure S14.

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525

526 **Growth curves**

527 *Nannochloropsis* species were maintained in flasks with f/2 media under 100 µmol m^{-2} s^{-1}
528 constant white light and entrained under diel conditions (12 h light/12 h dark, at 40 or 100 µmol
529 m^{-2} s^{-1}) for at least 10 days. Each culture was then diluted to make 3 50-mL cultures at the same
530 cell density and these diluted cultures were grown in the same diel conditions for 24 h before cell
531 counting began. Cells were counted using a Beckman-Coulter Z2 particle counter.

532

533 **Generation of luciferase expressing lines**

534 To generate the pNOC-LUC vector series (Figure S15, Dataset 1) the Gateway-luciferase
535 cassette from pMDC-LUC+HA (Farre and Kay 2007) and the hygromycin resistance cassette
536 from pSELECT100 (Vieler *et al.,* 2012) were PCR amplified and combined by Gibson
537 Assembly. The LDSP terminator (CCMP1779_4188) was PCR amplified from *N. oceanica*
538 CCMP1779 genome using the primers LDSP term sac F+ and LDSP term afl R- (Figure S16),
539 and digested with SacI and AflII to replace the 35S terminator as the terminator for the luciferase
540 gene. The LUC+HA gene was then replaced by either *N. oceanica* CCMP1779 codon optimized
541 coding sequences for firefly luciferase (FLUC) and, nanoluciferase (NLUC) (Hall *et al.,* 2012,
542 Poliner *et al.,* 2018b) or a Chlamydomonas codon optimized renilla luciferase (RLUC) (Ferrante
543 *et al.,* 2008) to form pNOC-Fluc, pNOC-Nluc, and pNOC-Rluc respectively using AscI and SacI
544 and the primers listed in Figure S16. The pNOC vectors were used to transform *N. oceanica*
545 CCMP1779. Since *N. salina* CCMP537 is resistant to hygromycin, the hygromycin resistance
546 cassette from the pNOC-GW vectors was replaced by a zeocin resistance cassette (BleC). This
547 cassette, which includes the CCMP537 EF promoter
548 (NODE_7115_length_93819_cov_37.515949:78382..79716), the zeocin resistance gene and the
549 CCMP1779 LDSP terminator, from *N. salina* selection construct pNSA-511 were cloned by
550 restriction digest (AhdI/SbfI) generating the pNSA-FLUC and vector (Dataset 1). The pNSA-511
551 construct was generated by cloning the CCMP537 EF promoter into pNOC-411 using SnabI and
552 XhoI (Poliner *et al.,* 2018b). The promoters of the cellulose synthase (NannoCCMP1779|5780,
553 NODE_11401_length_57650_cov_57.237450:4263..6099), *LHC1*
554 (NODE_14101_length_22726_cov_35.737923:17540..18097), and *LHC8*
555 (NannoCCMP1779|6809, NODE_11394_length_43512_cov_40.897591:1551..2663) promoters

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556 were amplified from the *N. oceanica* CCMP1779 and *N. salina* CCMP537 genomes using
557 primers listed in Figure S16 and inserted into pENTR-D-Topo (Invitrogen). The promoters were
558 transferred into luciferase reporter destination vectors by an LR clonase reaction (Invitrogen) and
559 used for transformation.
560
561 ***Nannochloropsis* transformation**
562 Promoter reporter vectors were linearized by restriction digest, concentrated by ethanol
563 precipitation, and resuspended in water. Transformations were conducted as described previously
564 (Poliner *et al.,* 2018c).
565
566 **Immunoblotting**
567 Reporter line 50 ml cultures in f/2 medium were grown in 250 ml flasks under 12 h /12 h
568 light/dark cycles, 200 µmol m^{-2} s^{-1}. Every 3 h, 5 ml aliquots were collected by centrifugation at
569 4,000 x G for 5 min, after decanting, pellets were transferred to 2 ml round bottom tubes and
570 centrifuged at 13,000 x G for 30 seconds. After aspiration of the supernatant, pellets were frozen
571 in liquid nitrogen. Frozen pellets were ground with 3 x 2 mm steel balls at 30 Hz for 2 minutes,
572 and resuspended in protein extraction buffer (100mM Tris pH 8.0, 2mM PMSF, 2% β-
573 mercaptoethanol, 4% SDS), heated at 80°C for 3 minutes, and centrifuged at 13,000 x G for 30
574 seconds. The protein concentration was determined using a RCDC protein quantification kit
575 (Bio-rad) and equal quantities (50 µg) of protein were loaded chronologically for the light/dark
576 timecourse, separated through 3-10% SDS-PAGE and transferred to PVDF membranes. The
577 FLUC-HA and NLUC-HA proteins were detected with anti HA-HRP antibody (Roche 3F10)
578 (1:1000) in TBST 5% milk. RLUC was detected with a primary anti-RLUC (MBL Life Sciences
579 PM047) 1:2000 in TBST 5% BSA and a secondary anti-Rabbit IgG-HRP (Bio-Rad 170-6515;
580 1:10000) antibody in TBST 5% milk. FLUC and RLUC immunoblots were visualized with
581 Clarity (Bio-rad) and NLUC visualized with Femto (Thermo Scientific) chemiluminescence
582 solutions. After visualization immunoblots were stained with Direct Blue 71 protein stain (Hee-
583 Youn Hong 2000).
584
585 **Luminescence assays**

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586 *Nannochloropsis* luciferase expressing lines were maintained and entrained under 12 h/12 h
587 light/dark cycles (200 µmol m^{-2} s^{-1}) if not otherwise indicated in the figure legends.
588 Luminescence assays were set-up in 96-well plates with 1.5 million cells in 200 µl and 500 µM
589 firefly luciferin (GoldBio), 100X dilution of renilla luciferin (Promega, E2920 Dual-Glo® Stop
590 & Glo® Reagent), or 10,000X dilution of NanoLuc substrate (Promega, N1110) in f/2 medium
591 per well. Luminescence assays were conducted with a Centro XS3 LB960 luminometer
592 (Berthold Technologies) over a 0.3 sec exposure and measurements were collected every hour
593 using MikroLab software. Bioluminescence experiments include independent cultures from 2-6
594 (*N. salina* CCMP537) or 3-7 (*N. oceanica* CCMP1779) independent transgenic lines (as
595 indicated in the figure legend), with the exception of the phase response curve for which data
596 from cultures from one transgenic *N. salina* CCMP537 *CS::FLUC* line is shown.
597
598 The casein kinase inhibitor PF-670462 (Cayman Chemicals) was resuspended in water at 2 mg
599 ml^{-1}. Pharmacological treatments were prepared using the protocol described above with some
600 modifications. Replicate wells with equal cell quantities for each treatment and mock were
601 prepared. Dilutions of the drug at twice the final intended concentration were prepared in the
602 luciferin f/2 solution and 100 µl added to the respective wells containing 100 µl of cells in f/2.
603
604 For substrate refeeding experiments cultures were entrained and maintained under light/dark
605 cycles. On the day of the experiment duplicate wells with equal cell densities and luciferin were
606 prepared. Luminescence measurements were collected every hour. After 48 h in order to restore
607 the starting concentration of substrate, 20 µl of f/2 and 10X concentrated substrate (10 mM
608 firefly luciferin, 1,000X dilution of NanoLuc Dual-Glo reagent, Promega) were added to the
609 respective wells.
610

611 **Statistical analyses**

612 Rhythmicity was quantified using tools on Biodare 2 (https://biodare2.ed.ac.uk/)(Moore *et al.,*
613 2014). If not otherwise indicated in the figure legend, the raw bioluminescence traces were first
614 detrended by subtracting a +12/-12 h moving average. To correct for damping effects, each of
615 the resulted data points was divided by the standard deviation of the corresponding +12/-12 h
616 moving window. This procedure is similar as one previously reported for damping circadian

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617 oscillations (Izumo *et al.,* 2006). Period length was estimated using FFT-NLS on the detrended
618 data (Plautz *et al.,* 1997). We defined a trace as rhythmic with a circadian type period when the
619 period length estimated with FFT-NLS was within 3 h of the estimate provided by either Enright
620 Periodogram (ERP)(Enright 1965) or Maximum Entropy Spectral Analysis (MESA) (Burg
621 1972), and the FFT-NLLS Relative Amplitude Error was < 0.7, the Period Error < 2 and the
622 Goodness Of Fit < 0.8. A good fit was further confirmed by visual inspection of the trace. For T6
623 experiments, traces periods of ~ 6h were considered "not rhythmic", since there was not apparent
624 circadian component. Other statistical analyses were implemented in GraphPad Prism.
625

626 **Phylogenetic analysis**

627 Protein sequences derived from the *N. oceanica* CCMP1779 and *N. salina* gene models and
628 related protein sequences were used to generate a multiple sequence alignment using the
629 Molecular Evolutionary Genetics Analysis 7 (MEGA7) program (Kumar *et al.,* 2016) and the
630 Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar 2004). The
631 alignment file was used to create a neighbor-joining method phylogenetic tree with 1000 rounds
632 of bootstrapping.
633

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636 the cell counter, and Stephanie Taylor for discussions and advice on the analyses of cyclic
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639 conflict of interest to declare.
640

641 **Short legends for supporting information**

642 **Figure S1.** Collection location of the *Nannochloropsis* strains used in this study.
643 **Figure S2.** Cell division in *Nannochloropsis* species under light/dark and constant light
644 conditions.
645 **Figure S3.** Effect of refeeding luciferase substrates on *in vivo* luminescence in *N. oceanica*
646 CCMP1779.
647 **Figure S4.** Overview of the period estimation analyses using Biodare2.

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648 **Figure S5**. *In vivo* bioluminescence of *CS::FLUC* expressing lines in constant dark.
649 **Figure S6.** Rhythms of *N. oceanica CS::FLUC* expression under constant light under different
650 temperatures
651 **Figure S7**. Bioluminescence rhythms under T6 cycles.
652 **Figure S8.** Phase response curve of *CS::FLUC* rhythms *in N. salina* under blue or red light.
653 **Figure S9.** CCT protein domains across taxa.
654 **Figure S10**. Expression of *N. oceanica* CCMP1779 CCT and bHLH-PAS genes under diel
655 cycles.
656 **Figure S11.** Phylogenetic analysis of *N. oceanica* (CCMP1779) and *N. salina* (CCMP537)
657 cryptochrome/photolyase proteins.
658 **Figure S12.** Comparison of bHLH-PAS domains across taxa.
659 **Figure S13**. Modulation of *CS::FLUC* rhythms by a CK1ε/δ inhibitor.
660 **Figure S14.** Spectrum of red and blue light sources.
661 **Figure S15.** Graphical representation of luciferase reporter vectors for *Nannochloropsis* species.
662 **Figure S16**. Primers used in this study.
663 **Dataset 1.** Plasmid sequences of constructs used in this study.
664
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992
993 **Figure legends**

994 **Figure 1.** Cell division oscillations in *Nannochloropsis* species under light/dark and constant
995 light conditions. (**a)** Cultures entrained under diel conditions (12 h light/12 h dark, 100 µmol m^{-2}
996 s^{-1}) and then moved to constant light (40 µmol m^{-2} s^{-1}). Values are the average ± SEM (n=3
997 cultures) and traces are nudged to aid visualization. (**b)** Cultures grown under diel conditions (12
998 h light/12 h dark, 40 µmol m^{-2} s^{-1}) for 10 days and then released to constant light (40 µmol m^{-2} s^{-1}). Values are the average ± SEM (n=3, *N. salina*; n=2 *N. oceanica*). Grey shading indicates dark
1000 period. **(c)** Residuals after determining a linear regression of the plots shown in (b) to extract the
1001 oscillatory component.

1002

1003 **Figure 2.** The development of bioluminescence reporters in *N. oceanica* CCMP1779. (**a)** *In vivo*
1004 luminescence oscillations of cells expressing *CS::RLUC*, *CS::NLUC, CS::FLUC* or
1005 *LHC8::FLUC* bioluminescence reporters under light/dark cycles. Dark grey shading indicates
1006 dark period. Luminescence was recorded every hour and the average bioluminescence per
1007 independent transgenic line ± SEM is shown (n=2 cultures). *CS*, *cellulose synthase* promoter;
1008 *LHC8*, *light harvesting complex 8* promoter; *RLUC*, *renilla luciferase*; *NLUC*, *nanoluciferase*;
1009 *FLUC*, firefly luciferase. (**b)** Representative western blots of the luciferase proteins from the
1010 transgenic reporter lines shown in (**a)** (top panel) and the blot stained with DB71 as loading
1011 control (bottom panel) over the course of one light/dark cycle. Light and dark periods are as
1012 indicated with grey shading in **c**. NLUC and FLUC contain a HA tag and were detected using an
1013 α-HA antibody. Renilla luciferase was detected using a specific antibody. **(c)** Quantitation of
1014 luciferase protein, and *in vivo* luminescence of one of the lines shown in (a), and the transcript
1015 abundance of the respective endogenous gene. Expression was normalized between 0 and 1 and
1016 the average ± SEM (n= 2 cultures) is shown. Transcript abundance data is from RNA-seq
1017 (Poliner *et al.,* 2015).

1018

1019 **Figure 3.** Bioluminescence oscillations of *N. salina* expressing *CS::FLUC* or *LHC8::FLUC*
1020 under light/dark cycles. Grey shading indicates dark period. Luminescence was recorded every

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1022 *CS*, *cellulose synthase* promoter; *LHC8*, *light harvesting complex 8* promoter; *FLUC*, *firefly*
1023 *luciferase.*
1024
1025 **Figure 4**. *In vivo* bioluminescence oscillations of *CS::FLUC* expressing lines from two
1026 *Nannochloropsis* species in constant light. Cultures were entrained under cycles of 12 h light/12
1027 h dark (200 µmol m^{-2} s^{-1}) and 22°C and released under either constant white (**a,b**), red (**c,d**) or
1028 blue light (**e,f**) at the indicated intensities (100 µmol m^{-2} s^{-1}, magenta; 40 µmol m^{-2} s^{-1}, blue; 10
1029 µmol m^{-2} s^{-1}, black). Right panels represent the average of detrended bioluminescence traces ±
1030 SEM (*N. salina* n=4-18 cultures, two independent lines; *N. oceanica* n=6-20, three independent
1031 lines). Only rhythmic traces are shown, with the exception of conditions that resulted in no
1032 rhythmic traces, for which the average trace is shown as a dotted line. Percent values indicate
1033 percent rhythmic traces. Traces are nudged to aid visualization. Left panels represent the period
1034 length estimate by FFT-NLLS on Biodare 2 using data from the respective right panel, only
1035 period lengths of traces considered rhythmic are shown, line indicates average.
1036
1037 **Figure 5**. Rhythms of *N. salina CS::FLUC* expression under different temperatures. Cultures
1038 were entrained under cycles of 12 h light/12 h dark (200 µmol m^{-2} s^{-1}) and 22°C. (**a)** *In vivo*
1039 bioluminescence under constant white light conditions (10 µmol m^{-2} s^{-1}). Cultures were switched
1040 to 17°C or 19°C at time 12 h, and to 25°C or 28°C at time 24 h. The average detrended
1041 bioluminescence of rhythmic cultures ± SEM is shown (*N. salina* n=2-15, two independent lines;
1042 *N. oceanica* n=6-18, three independent transgenic lines). Traces are nudged to aid visualization.
1043 (**b)** Period lengths estimated by FFT-NLLS on Biodare 2 using data from *N. salina* shown in (**a)**.
1044 Only period lengths of traces considered rhythmic are shown. (**c)** *In vivo* bioluminescence of
1045 *CS::FLUC* in *N. salina* under T6 cycles. Cultures were transferred to cycles of 3 h white light
1046 (100 µmol m^{-2} s^{-1}) and 3 h of dark at time 24 h. Grey shadings indicate dark periods. Cultures
1047 were switched to different temperatures as described for (**a)**. The average bioluminescence per
1048 rhythmic culture ± SEM of rhythmic traces is shown (n=2-7). Percent values indicate the percent
1049 of rhythmic traces. **(d)** Period length of traces shown in **c** estimated by FFT-NLLS on Biodare 2.
1050 Q_{10}, factor by which the rate of a reaction varies in response to a 10°C change in temperature
1051 calculated using the slopes from (**b)** and (**d)** respectively.

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1052

1053 **Figure 6.** Light entrainment in *Nannochloropsis* species using *CS::FLUC* expressing lines. (**a**)
1054 Experimental jet-lag recovery in *N. salina*. Left panel, *in vivo* bioluminescence, traces from one
1055 representative experiment with cultures from two independent transgenic lines are shown. Grey
1056 shading indicates dark period. Cells were entrained under cycles of 12 h light/12 h dark and 22°C
1057 before the start of the experiment. At time 72 h (arrow) the night was extended by 6 h. Right
1058 panel, time of maximum luminescence with respect to last dawn (ZT in h) before (day 1 and 2)
1059 and after (days 3-4) the experimental jet-lag (average ± SEM; n= 19). (**b-d)** Phase response curve
1060 of *CS::FLUC* rhythms *in N. salina*. After entrainment under cycles of 12 h light/12 h dark and
1061 22°C for 7 days, cultures were exposed to one dark period of variable length before being
1062 transferred to constant light (10 µmol m^{-2} s^{-1}). Representative experiment using white light and
1063 extended nights (**b)** or short nights **(c)** (average ± SEM, n = 4, one transgenic line). For **(b)** the
1064 length of the dark periods were 12 h (black), 15 h (magenta), 18 h (blue), 21 h (yellow) or 24 h
1065 (green); and for **(c)**, 0 h (black), 3 h (magenta), 6 h (blue), 9 h (yellow) or 12 h (green). **(d)** Phase
1066 of peak luminescence (x-axis) versus the duration of the dark period (y-axis) after transfer to
1067 either white, blue or red light (average ± range of two independent transgenic lines). **(e-f)** *In vivo*
1068 luminescence under different T-cycles, with 30 µmol m^{-2} s^{-1} white light (average ± SEM, n = 16,
1069 two transgenic lines). **(g-h)** Phase angle of bioluminescence oscillations shown in (e) and (f),
1070 using traces from the second day in the respective T-cycle (average ± SEM, n = 16). Significant
1071 difference to T24 (*) $p<0.05$, (**) $p<0.001$(unpaired *t*-test).

1072

1073 **Figure 7.** Temperature entrainment in *Nannochloropsis* species. **(a)** *In vivo* bioluminescence
1074 rhythms of *CS::FLUC* in *N. oceanica* (black) and *N. salina* (blue). Cultures were entrained under
1075 cycles of 12 h 17°C /12 h 27°C and 200 µmol m^{-2} s^{-1} white light. Bioluminescence was recorded
1076 for one warm/cool cycle (40 µmol m^{-2} s^{-1}) and at time 24 h cells were transferred to constant
1077 temperature (22°C) and constant light (40 µmol m^{-2} s^{-1}). The detrended bioluminescence ± SEM
1078 of cycling lines (*N. salina* n=6, two independent lines; *N. oceanica* n=3, three independent lines);
1079 purple shading indicates cool period, dotted lines indicate subjective cool to warm transition.
1080 Amplitude and baseline detrending was implemented on Biodare 2. **(b)** Period length of cycling
1081 cultures estimated using FFT-NLLS on Biodare 2. Percent cycling cultures are indicated.

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1083 **Figure 8.** Modulation of *CS::FLUC* rhythms in *N. salina* by a CK1ε/δ inhibitor. (**a**) *In vivo*
1084 bioluminescence rhythms of *N. salina CS::FLUC* expressing lines. The average ± SEM from
1085 rhythmic traces is shown (n =3-8, two independent lines). Cultures were entrained under cycles
1086 of 12 h light/12 h dark (200 µmol m^{-2} s^{-1}) and released to constant light (10 µmol m^{-2} s^{-1}) at time
1087 24 h. Cells were treated with PF-670462 at the indicated concentrations at time 12 h. Traces from
1088 one representative experiment are shown. Dark grey shading indicates dark period, light grey
1089 shading indicates subjective dark. **b** Time of first peak of *CS::FLUC* luminescence (n=8-16)
1090 from two independent experiments. * Indicate a significant difference with the vehicle control
1091 (one-way ANOVA with Dunnett's post hoc test, α=0.05).
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Figures

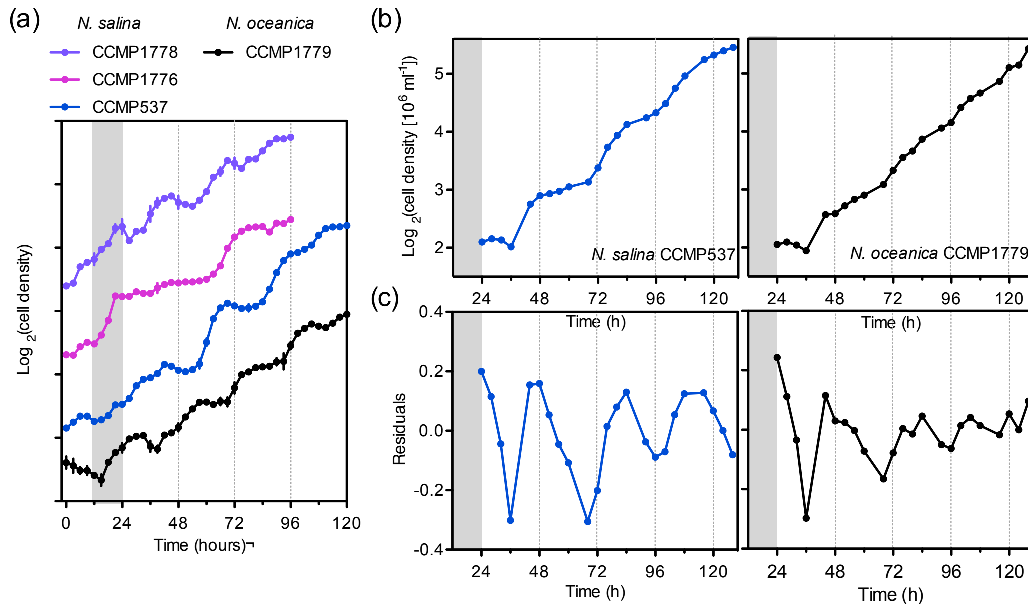

Figure 1. Cell division oscillations in *Nannochloropsis* species under light/dark and constant light conditions. **(a)** Cultures entrained under diel conditions (12 h light/12 h dark, 100 µmol m^{-2} s^{-1}) and then moved to constant light (40 µmol m^{-2} s^{-1}). Values are the average ± SEM (n=3 cultures) and traces are nudged to aid visualization. **(b)** Cultures grown under diel conditions (12 h light/12 h dark, 40 µmol m^{-2} s^{-1}) for 10 days and then released to constant light (40 µmol m^{-2} s^{-1}). Values are the average ± SEM (n=3, *N. salina*; n=2 *N. oceanica*). Grey shading indicates dark period. **(c)** Residuals after determining a linear regression of the plots shown in (b) to extract the oscillatory component.

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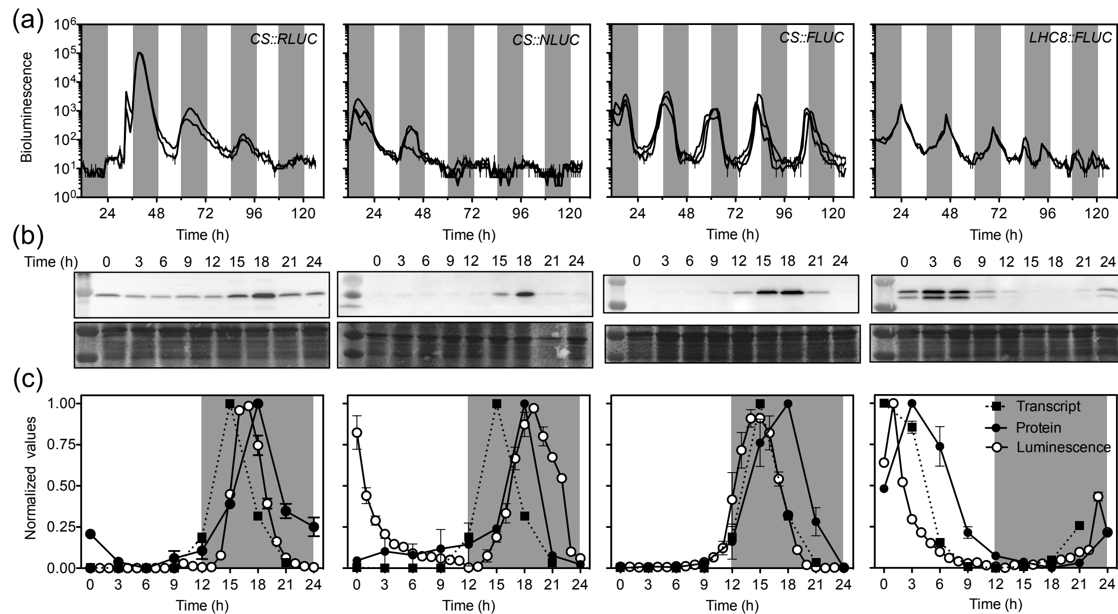

Figure 2. The development of bioluminescence reporters in *N. oceanica* CCMP1779. **(a)** *In vivo* luminescence oscillations of cells expressing *CS::RLUC*, *CS::NLUC, CS::FLUC* or *LHC8::FLUC* bioluminescence reporters under light/dark cycles. Dark grey shading indicates dark period. Luminescence was recorded every hour and the average bioluminescence per independent transgenic line ± SEM is shown (n=2 cultures). *CS*, *cellulose synthase* promoter; *LHC8*, *light harvesting complex 8* promoter; *RLUC*, *renilla luciferase*; *NLUC*, *nanoluciferase*; *FLUC*, firefly luciferase. **(b)** Representative western blots of the luciferase proteins from the transgenic reporter lines shown in **(a)** (top panel) and the blot stained with DB71 as loading control (bottom panel) over the course of one light/dark cycle. Light and dark periods are as indicated with grey shading in **c**. NLUC and FLUC contain a HA tag and were detected using an α-HA antibody. Renilla luciferase was detected using a specific antibody. **(c)** Quantitation of luciferase protein, and *in vivo* luminescence of one of the lines shown in (a), and the transcript abundance of the respective endogenous gene. Expression was normalized between 0 and 1 and the average ± SEM (n= 2 cultures) is shown. Transcript abundance data is from RNA-seq (Poliner *et al.,* 2015).

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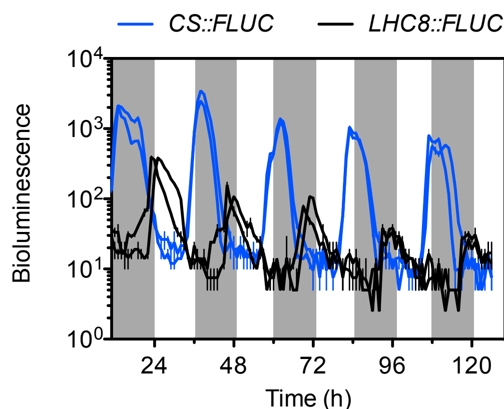

Figure 3. Bioluminescence oscillations of *N. salina* expressing *CS::FLUC* or *LHC8::FLUC* under light/dark cycles. Grey shading indicates dark period. Luminescence was recorded every hour and the average bioluminescence per independent transgenic line ± SEM is shown (n=2). *CS*, *cellulose synthase* promoter; *LHC8*, *light harvesting complex 8* promoter; *FLUC*, *firefly luciferase.*

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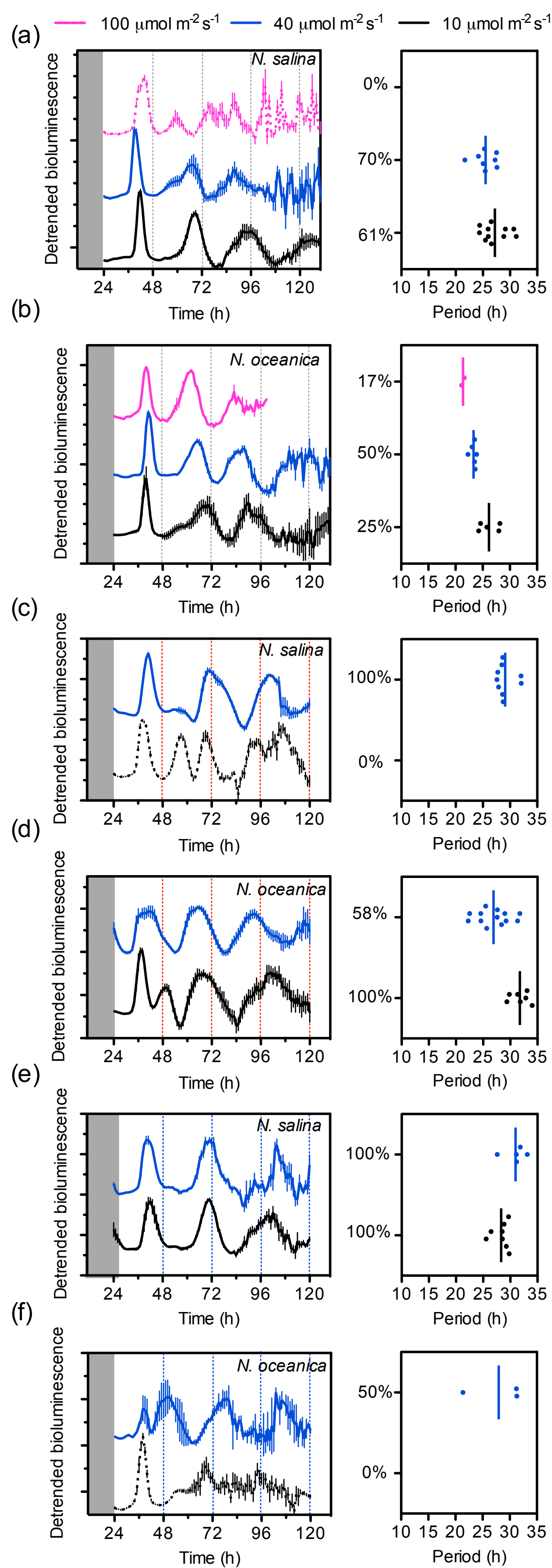

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Figure 4. *In vivo* bioluminescence oscillations of *CS::FLUC* expressing lines from two *Nannochloropsis* species in constant light. Cultures were entrained under cycles of 12 h light/12 h dark (200 µmol m^{-2} s^{-1}) and 22°C and released under either constant white **(a,b)**, red **(c,d)** or blue light **(e,f)** at the indicated intensities (100 µmol m^{-2} s^{-1}, magenta; 40 µmol m^{-2} s^{-1}, blue; 10 µmol m^{-2} s^{-1}, black). Right panels represent the average of detrended bioluminescence traces ± SEM (*N. salina* n=4-18 cultures, two independent lines; *N. oceanica* n=6-20, three independent lines). Only rhythmic traces are shown, with the exception of conditions that resulted in no rhythmic traces, for which the average trace is shown as a dotted line. Percent values indicate percent rhythmic traces. Traces are nudged to aid visualization. Left panels represent the period length estimate by FFT-NLLS on Biodare 2 using data from the respective right panel, only period lengths of traces considered rhythmic are shown, line indicates average.

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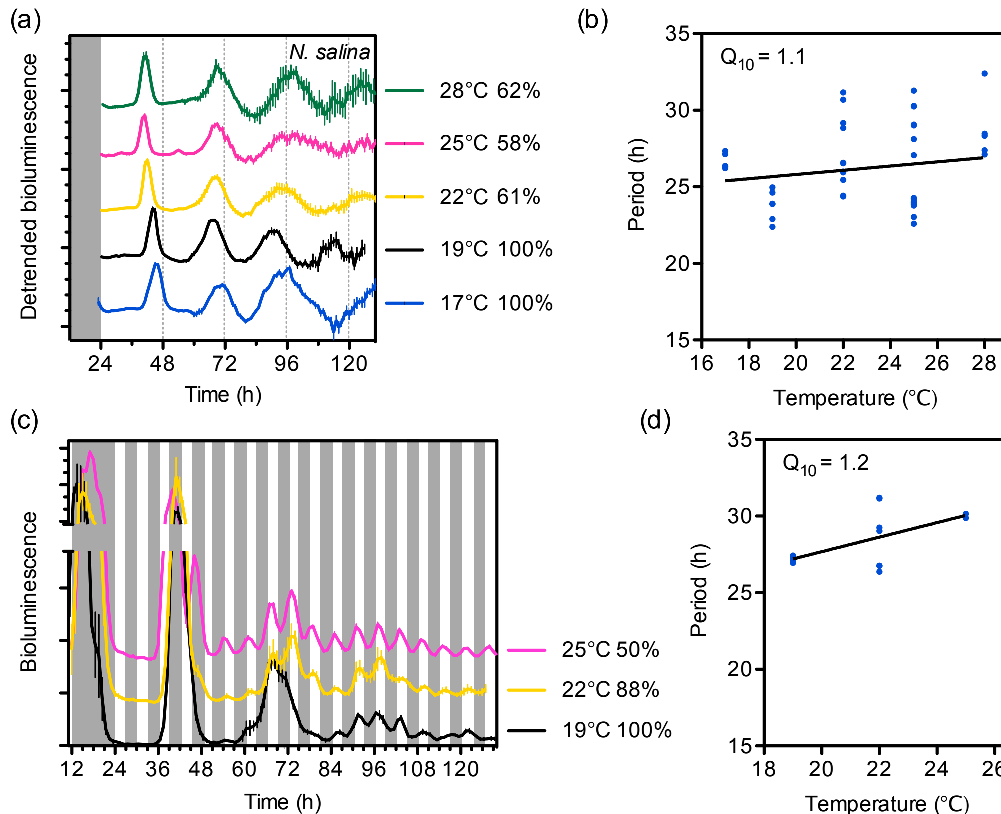

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Q_{10}, factor by which the rate of a reaction varies in response to a 10°C change in temperature calculated using the slopes from **(b)** and **(d)** respectively.

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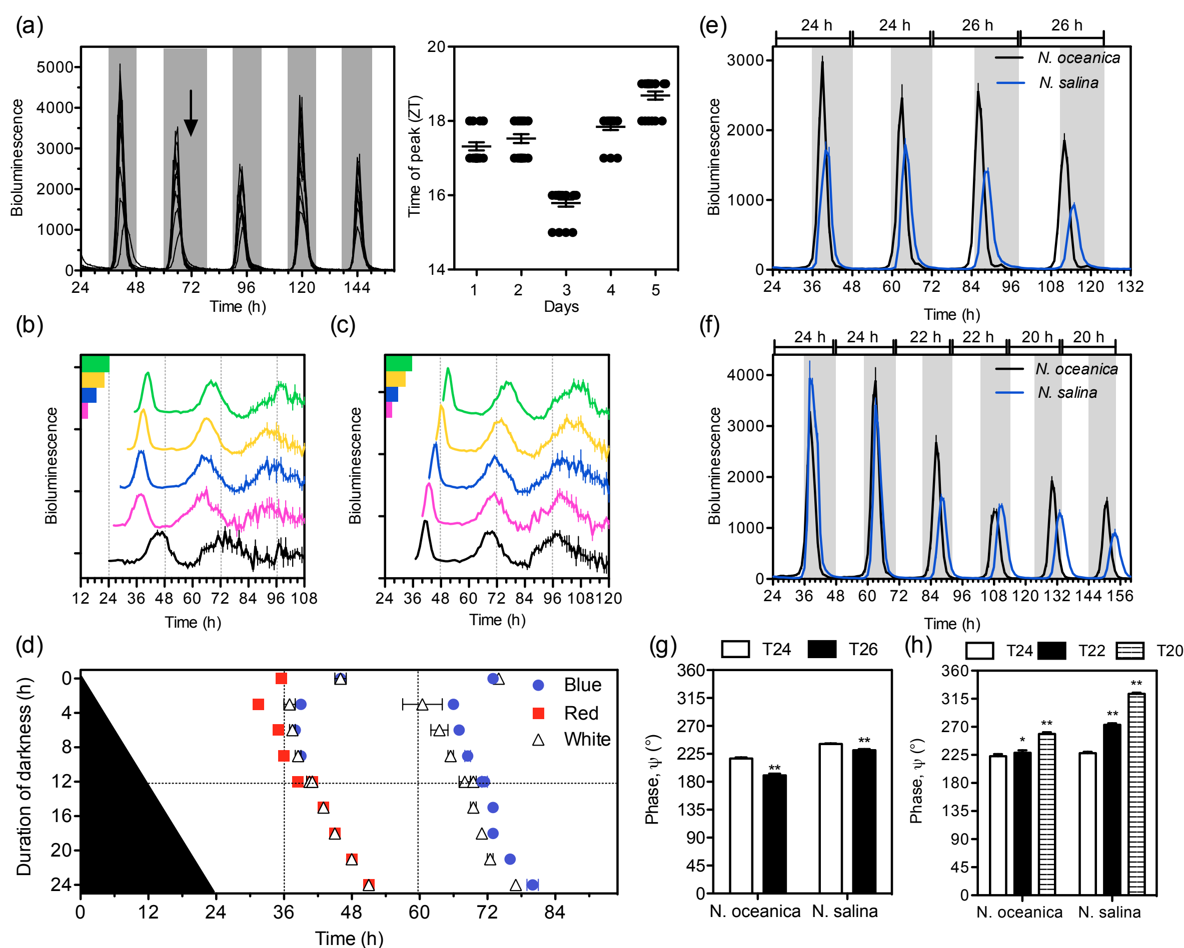

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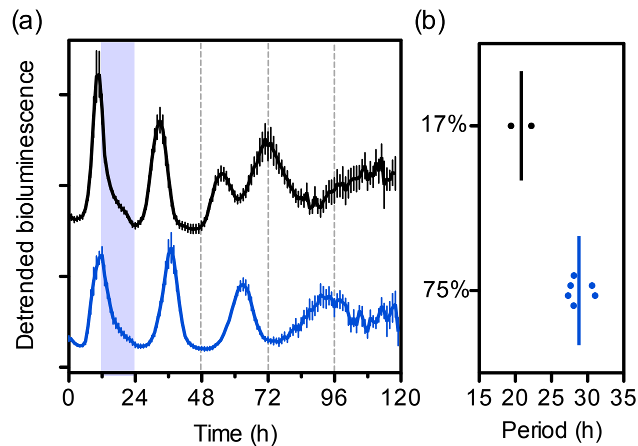

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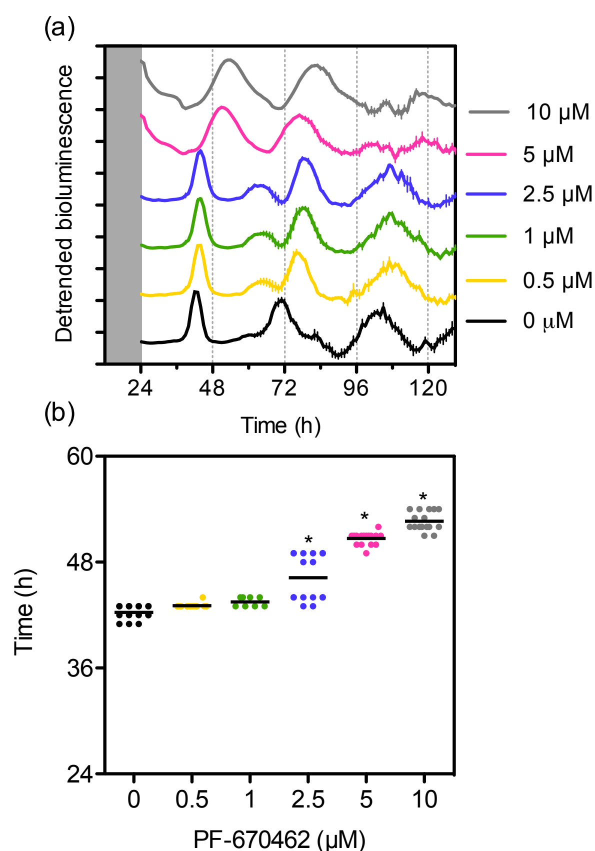

Figure 8. Modulation of *CS::FLUC* rhythms in *N. salina* by a CK1ε/δ inhibitor. **(a)** *In vivo* bioluminescence rhythms of *N. salina CS::FLUC* expressing lines. The average ± SEM from rhythmic traces is shown (n =3-8, two independent lines). Cultures were entrained under cycles of 12 h light/12 h dark (200 µmol m^{-2} s^{-1}) and released to constant light (10 µmol m^{-2} s^{-1}) at time 24 h. Cells were treated with PF-670462 at the indicated concentrations at time 12 h. Traces from one representative experiment are shown. Dark grey shading indicates dark period, light grey shading indicates subjective dark. **(b)** Time of first peak of *CS::FLUC* luminescence (n=8-16) from two independent experiments. * Indicate a significant difference with the vehicle control (one-way ANOVA with Dunnett's post hoc test, α=0.05).