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1 **Triacylglyceride production and autophagous responses in *Chlamydomonas reinhardtii***
2 **depend on resource allocation and carbon source**
3
4 **Running title:** Lipid productivity in *Chlamydomonas reinhardtii*
5
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25 **Abbreviations: FAME, Fatty Acid Methyl Ester; FFA, Free fatty acid; GC-FID, Gas-**
26 **Chromatography-Flame Ionisation Detection; GC-MS, Gas-Chromatography-Mass**
27 **Spectrometry; TAG, Triacylglyceride**
28
29 **Key words:** **lipid synthesis, algae, biofuel, triacylglycerides, gas chromatography**
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35 **Abstract:**

36 To improve the economic viability of microalgal biodiesel, it will be essential to optimize
37 productivity of fuel molecules such as triacylglyceride (TAG) within the microalgal cell. To
38 understand some of the triggers required for the metabolic switch to TAG production, we
39 studied the effect of carbon supply (acetate or CO_2) in *Chlamydomonas reinhardtii* (wild type
40 and the starch-less mutant *sta6*) grown under low N availability. As expected, initial rates of
41 TAG production were much higher when acetate was present, compared to strictly
42 photosynthetic conditions, particularly for *sta6,* which cannot allocate resources to starch.
43 However, in both strains TAG production plateaued after a few days in mixotrophic cultures,
44 whereas under autotrophic conditions TAG levels continued to rise. Moreover, the reduced
45 growth of *sta6* meant that the greatest productivity (measured as mg TAG L^{-1} day^{-1}) was
46 found in wild type growing autotrophically. Wild type cells responded to low N by
47 autophagy, as shown by degradation of polar (membrane) lipids and loss of photosynthetic
48 pigments, and this was less in cells supplied with acetate. In contrast little or no autophagy
49 was observed in *sta6* cells regardless of carbon supply. Instead, very high levels of free fatty
50 acids were observed in *sta6*, suggesting considerable alteration in metabolism. These
51 measurements show the importance of carbon supply and strain selection for lipid
52 productivity. Our findings will be of use for industrial cultivation, where it will be preferable
53 to use fast-growing wild type strains supplied with gaseous CO_2 under autotrophic
54 conditions, rather than requiring an exogenous supply of organic carbon.
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62 **INTRODUCTION**

63

64 Microalgae have the potential to produce significant amounts of neutral lipids, such as
65 triacylglyceride (TAG), which can be used as a source of biodiesel (1). In species such as
66 *Chlorella vulgaris* (2), *Nannochloropsis oculata* (3) and *Chlamydomonas reinhardtii* (4-7)
67 TAGs can accumulate up to 40-50% dry weight. To initiate the desired TAG production a
68 nutrient limitation regime is usually required, such as removal of nitrogen (N) from the
69 growth medium (8). Nutrient limitation also results in a substantial increase in the amount of
70 starch (6, 7, 9), but in mutant strains of *C. reinhardtii* that are unable to synthesise starch,
71 excess photosynthate cannot be allocated to this storage molecule, so much higher levels
72 TAG per cell than for wild type have been reported (10-12). For example, there is a 30-fold
73 increase in the number of TAG lipid droplets in *C. reinhardtii sta6*, which is deficient in
74 ADP-glucose pyrophosphorylase (13). In all cases however, such TAG production comes at a
75 cost, as the growth rate of the cultures can also be substantially decreased by nutrient
76 limitation (6, 10-12). This situation can be avoided to some extent by reducing, rather than
77 removing, the N from the growth medium as optimal TAG production in *Chlorella* was seen
78 when cells were grown in low N containing medium, rather than in complete absence of N
79 (2). Nevertheless, in order to improve the economic cost of algal biofuels it is essential to
80 understand how changes in the availability of nutrients to the algal cells can influence the
81 triggers that underpin TAG metabolism and production, so as to optimise productivity.

82 Studies of lipid production in microalgae have shown that, as well as changes in N
83 availability, addition of organic carbon to the medium can result in the cells producing
84 greater amounts of lipids and rates of growth than N-limitation alone (14, 15). For example,
85 by supplementing medium with 1% acetate *Chlorella vulgaris* can increase its total lipid
86 content from 33 to 36 % of its dry weight when grown under N limitation (16). These and

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87 other observations have led to the presumption that TAG biosynthesis is responsive to the
88 C/N ratio that cells experience, such that there is a switch between growth when the ratio is
89 normal, to TAG production when it is unbalanced by reducing the N, or by increasing the C,
90 or both.

91 Under N-limitation, it has been postulated that autophagy takes place, such that
92 internal (probably thylakoid) membranes are broken down and used to provide the precursors
93 for TAG biosynthesis. This conclusion is supported by studies that show cells grown in N-
94 depleted medium had decreased concentrations of polar lipids and chlorophyll (6, 7, 9, 12,
95 17). In fact, the majority of studies on TAG accumulation in *C. reinhardtii* include fixed
96 carbon in the form of acetate in the medium (7, 12, 13, 18), under which conditions
97 photosynthetic activity is reduced (12). The utilisation of acetate for cellular metabolism is
98 likely to be via the glyoxylate cycle and gluconeogenesis, but for lipid synthesis it may well
99 be as a result of direct incorporation of acetyl CoA into fatty acids, as has been shown for
100 higher plant chloroplasts (19-21). The supply of acetate to *C. reinhardtii* could therefore
101 provide a preferential source of acetyl-CoA for *de novo* fatty acyl synthesis over that from
102 pyruvate derived from photosynthesis.

103 To enable economic production of algal biofuels, it would be preferable to use
104 gaseous CO_2 in autotrophic growth environments rather than supplying exogenous organic
105 carbon (22), which in any case can exacerbate problems with bacterial contamination (23). In
106 this paper, we sought to compare directly the effect of organic or inorganic carbon supply
107 (acetate or CO_2) on TAG production and autophagy in *C. reinhardtii* wild type and *sta6* cells
108 grown under low N availability. Specifically, we hypothesised that if acetate were being
109 directly used as a precursor for *de novo* fatty acid synthesis the initial rate of TAG production
110 would increase, and there would be less cellular autophagy, especially in *sta6* cells where
111 TAG production is greater than wild type cells. At the same time, we aimed to address the

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112 issue of whether it is the C/N ratio, rather than absolute concentrations, that is sensed by the
113 cells in determining whether TAG production is initiated. The potential productivity of these
114 strains in industrial settings is also addressed by considering TAG accumulation beyond the
115 short term (2 days) initial increase in TAG production investigated in other studies (8, 16).
116
117 **METHODS**
118
119 ***Culturing of algae**:* Stocks of *Chlamydomonas reinhardtii* WT-12 *nit-* (derived from strain
120 CC124 137c *mt*⁻ *nit1 nit2*) and *sta6* (also known as BAFJ5, (*cw15 arg7-7 nit1 nit2 sta6-*
121 *1::ARG7*)) were obtained from Dr Saul Purton (University College London) (24, 25). The
122 parental strain of the cell-wall deficient *sta6* is thought to be strain 330 (*mt*⁺ *arg7-7 cw15 nit1*
123 *nit2*) (7, 12, 24). We chose to use wild type-12 (hereinafter referred to as WT-12) in our
124 studies, since it is the parent to *cw15,* and has a complete cell-wall, which would be much
125 more likely to be suitable for large scale cultivation.
126
127 ***Colony growth on agar plates**:* Five µL spots of *C. reinhardtii* WT-12 or *sta6* (normalised to
128 cell number, Z2 particle count analyser, Beckman Coulter Ltd., UK) stock cultures were
129 added to either HSM or TAP solid 2 % agar plates. Plates were incubated at 25 ºC with either
130 continuous or diurnal (12 light/12 dark hours) light (120 µmol m^{-2} s^{-1}) for seven days.
131
132 ***Transfer of stock cultures to normal (1N) or low (0.1 N) nitrogen containing medium**:*
133 Cells were initially grown in either Sueoka's high salt medium (HSM) containing 9.34 mM
134 NH_4Cl (1N HSM) (26) for autotrophic growth or Tris-Acetate-Phosphate (TAP) containing
135 7.01 mM NH_4Cl (1N TAP) for mixotrophic growth (27). Cultures were placed in a closed
136 incubator (Infors HT Multitron incubator, Basel, Switzerland) and shaken at 120 rpm,

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137 continuous light (120 µmol m^{-2} s^{-1}) at 25 °C. Flasks containing HSM were aerated with 5%
138 CO_2 via a sterile filter. At an optical density (OD_{600}) (Thermo Spectronic UV1, Thermo
139 Scientific) of approximately 0.8, the cultures were centrifuged at 25 °C, 600 *g* for 10 min
140 (Beckman Coulter, Avanti J-26 XP). Pellets were washed with sterile water to remove excess
141 medium salts and re-centrifuged as above, then resuspended in the same volume of 4
142 different media: HSM containing 9.34 mM NH_4Cl (1N HSM), 0.934 mM NH_4Cl (0.1N
143 HSM), TAP containing 7.01 mM NH_4Cl (1N TAP) or 0.701 mM NH_4Cl (0.1N TAP).
144 Cultures were grown as above for 10 days from transfer (day 0), with individual flasks being
145 taken for analysis every other day. For each flask OD_{600} and OD_{750} and dry cell mass were
146 determined according to Stephenson et al. (2) with the rest of the sample being analysed for
147 the cellular constituents as below.
148
149 ***Extraction and analysis of total cellular lipids****:* All chemicals were purchased from Sigma
150 chemicals unless stated otherwise. Lipid extraction was based on a modified Bligh and Dyer
151 method (28) as outlined in Horst et al. (29). To extract the total lipids, cells from 5 mL of
152 culture were pelleted (600 *g*, 25 °C) for 15 min. The supernatant was removed and 10 mL of
153 chloroform:methanol (2:1, v/v) was added to the tube, vortexed (15 s) and sonicated for 30
154 min. Prior to extraction, samples were spiked with 1 mg mL^{-1} C15:0 fatty acid. Five mL
155 chloroform:methanol (2:1, v/v) and 5 mL of deionised water were then added to separate the
156 two phases. The lower chloroform phase was dried in a solvent evaporator (45 °C) (GeneVac
157 EZ-2, SP Scientific, Ipswich, UK) and resuspended in 200 µL *n*-heptane (27). To test
158 whether lipase activity was influencing the lipid profiles, especially the free fatty acids, a
159 lipase inhibitor (100 µL isopropanol) was added to the pelleted samples of WT-12 and *sta6*
160 cultures (day 8 of 0.1 N treatment in TAP) during storage at -80 ºC (30).
161

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162 TAGs, polar lipids and free fatty acids (FFA) in the crude total lipid extract were analysed by
163 gas chromatography-flame ionisation detection (GC-FID) with a Varion Select Biodiesel for
164 Glycerides GC metal column (10 m x 0.32 mm, 0.1 µm film thickness (Agilent Technology
165 part number CP9076) as described in Horst et al. (29). Free fatty acids were identified by co-
166 retention of fatty acid standards and quantified using standard curves derived from C17:0
167 heptadecanoic acid (0.075-1.2 mg mL^{-1}). Polar lipids were identified by co-retention of
168 monogalactosyl diglyceride, digalactosyl diglyceride, dioleyl phosphatidyl, phosphatidic
169 acid, phosphatidic glycerol and sulphoquinovosyl diglyceride (Lipid Products, South
170 Nutfield, UK) and quantified using monogalactosyl diglyceride (0.25-1.0 mg mL^{-1}) as this is
171 reported to be the dominant polar lipid in thylakoid membranes (31). TAGs were quantified
172 using glyceryl tripalmitate (0.0-0.25 mg mL^{-1}) (Supplementary figure 1).
173
174 ***Preparation, identification and quantification of fatty acid methyl esters (FAMEs)**:* The
175 total lipid fractions of the samples were converted to FAMEs (acid catalysed) as described in
176 Stephenson et al. (2). The FAMEs were separated and identified using gas chromatography
177 (Thermo Scientific Trace GC Ultra) with a Zebron ZB-Wax Capillary GC column (30 m x
178 0.25 mm, 0.25 µm film thickness; Phenomenex, UK). The injection volume was 1 µL with a
179 35:1 split ratio, and an injector temperature of 230 ^{o}C, using helium as a carrier gas at a
180 constant flow of 1.2 mL min^{-1}. The following gradient was used: initial oven temperature 60
181 ^{o}C, 2 min; 150 ^{o}C at 15 ^{o}C min^{-1}; 230 ^{o}C at 3.4 ^{o}C min^{-1}. Detector temperature was 250 ^{o}C.
182 FAMEs were identified by co-elution with a FAME standard mix (Grain Fatty Acid Methyl
183 Ester Mix Sigma-Aldrich, cat no. 47801) and were quantified using standard curves derived
184 from C17:0 methyl esters.
185
186 ***Analysis of free fatty acids**:*

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187 Fifteen µl of total the lipid extract was spotted onto a glass-backed 20 x 20 cm silica gel 60
188 plate (Merck). The free fatty acids, polar lipids and TAGs were separated using a 100 mL
189 running solvent of hexane:diethyl ether:acetic acid (70:30:2, v/v/v) in a TLC developing tank.
190 Once run, the plate was dried under a stream of N_2 gas before being sprayed with 0.2% (w/v)
191 8-anilino-1-naphthalenesulphonic acid in methanol. Free fatty acids were visualised under
192 ultraviolet light (366 nm), scraped off the plate and converted to FAMEs as above.
193 They, alongside the underivatised free fatty acids in the crude total lipid extract, were further
194 analysed and identified by gas chromatography-mass spectrometry (Thermo Scientific Trace
195 GC Ultra – DSQII EI MS) with a Zebron ZB-FFAP GC column (30 m x 0.25 mm, 0.25 µm
196 film thickness; Phenomenex, UK). The injection volume was 1 µL with a 10:1 split ratio
197 (split flow 12 mL min^{-1}) using the same GC conditions as above (without FID). The mass
198 spectrometry conditions in the positive mode were: ion source, 250 ºC; mass range 45-650
199 Da; scan rate of 1783 amu/s. Free fatty acids were identified by co-retention with standards
200 (Sigma) and mass spectral search libraries (National Institute of Standards and Technology
201 NIST v2.0 http://www.nist.gov/srd/nist1a.cfm) and MassBank (32) (Mass spectra of
202 identified peaks are provided in the supplementary spreadsheet file).
203
204 ***Starch and pigment analysis***: Total starch was quantified using a commercial
205 amyloglucosidase enzymatic kit (Starch Assay Kit SA-20, Sigma-Aldrich) according to the
206 manufacturer's instructions. Total chlorophyll and carotenoid concentrations were
207 determined after extraction of cell pellet with dimethylformamide using the equations by
208 Inskeep and Bloom (33) and Wellburn (34), respectively.
209
210 ***Confocal imaging***: Prior to imaging, 5 µL of Nile Red (50 µg mL^{-1} acetone) was added to
211 0.5 mL of culture to stain neutral lipids (TAGs). The cells were visualised under a confocal

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212 fluorescence microscope (Leica DM6000B; scan head Leica TCS SP5) using the following
213 settings: 63 x 1.32 oil lens; excitation 488 nm at 40 %; emission 550-590 nm for Nile Red
214 and 685-730 nm for chlorophyll fluorescence.
215
216 ***Statistics***: Statistical significance of values between day 0 and 10 was determined by the T-
217 test function in Excel (Microsoft Office 2007).
218
219 **RESULTS**
220
221 ***Accumulation of lipids and starch in cells grown under nitrogen deficiency***
222
223 To study the effect on TAG production and autophagy of different C sources - either
224 autotrophic (HSM + CO_2) or mixotrophic (TAP, containing acetate) – *C. reinhardtii* WT-12
225 and *sta6* cells were grown to late exponential phase, pelleted, washed and transferred to fresh
226 medium with either normal N (1N), or low N (0.1N) under these two C regimes. Complete
227 removal of N from the medium results in abrupt cessation of cell growth (2), so to increase
228 TAG productivity reduced N is preferable (8). Over a 10 day period, Nile Red staining and
229 confocal microscopy revealed that cells grown in low N medium accumulated many large
230 neutral-lipid oil bodies that were not seen in algae grown in normal N (Fig. 1). These visual
231 changes were quantified by extracting the lipid and measuring TAG content by GC analysis,
232 and starch content was determined by enzymatic analysis of glucose after acid hydrolysis.
233 Figure 2 presents the results of TAG and starch levels in the cells over the time course
234 of N-deprivation. As expected, in 1N medium (solid lines), little or no TAG was produced
235 irrespective of the C source. In cells grown mixotrophically in 0.1N TAP medium, both WT-
236 12 and *sta6* produced TAGs over the time course (dashed lines), this increase was statistically

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237 significant between day 0 and 10 ($P < 0.01$). However, whereas in WT-12 TAG
238 accumulation occurred over the first two days and then stayed more or less constant, in *sta6*
239 the TAG increased for a longer period, and finally reached levels some four-fold higher than
240 WT-12 (161 versus 37 mg TAG g^{-1} dry cell weight). These values are in line with those of
241 others who found higher levels of lipid in starchless mutants compared to those with normal
242 starch content (7).

243 The profile of TAG production (and starch in WT-12) in cells grown autotrophically
244 (HSM + CO_2), was quite different (Fig. 2). In all conditions, after an initial lag of 2-3 days
245 there was continual synthesis throughout the time course ($P \leq 0.01$ between day 0 and 10),
246 and the total accumulated after 10 days in *sta6* was only slightly higher than in WT-12 (109
247 vs 84 mg TAG g^{-1} dry cell weight). This gradual increase in TAG accumulation in WT-12
248 continued to a concentration of 167 mg TAG g^{-1} dry cell weight after 15 days of growth in
249 0.1N HSM (data not shown).

250 Table 1 shows the data expressed as rate of TAG produced per unit time per g dry cell
251 weight or L of culture (ie. productivity), and it is clear that under autotrophic conditions TAG
252 production is more or less constant (~10-15 mg TAG g^{-1} dry weight day^{-1} from day 4 to 10),
253 whereas under mixotrophic conditions productivity ceases after the initial burst. However,
254 there is an important difference between the WT-12 and *sta6* strains, namely that, despite the
255 higher level of TAG per unit dry weight in *sta6*, the overall production of TAG in WT-12
256 cultures (expressed as mg TAG L^{-1} day^{-1}) is much greater than for the starchless mutant.

257 In the WT-12 strain 0.1N TAP and 0.1N HSM conditions also led to a significant
258 increase in starch content (P = 0.069 and P 0.006 respectively between day 0 and 10),(Fig. 2).
259 When the accumulation of TAG is plotted against starch levels (Fig. 3), the two storage
260 molecules show a linear relationship up to ~ 500 mg starch g^{-1} d.wt. Thereafter, in the cells

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261 growing in 0.1N HSM, the amount of TAG steadily increased, with little or no increase in
262 starch content, suggesting that in these conditions TAG and starch production is uncoupled.
263
264 ***Evidence of autophagy and de novo synthesis of fatty acyl groups for TAG production***
265
266 Our criteria for assessing autophagy in cells grown in 0.1N containing medium were: 1) a
267 decrease in chlorophyll and carotenoid (9, 35); 2) a decrease in levels of polar (ie membrane)
268 lipids with a concurrent increase in TAGs (36); and 3) no change in total fatty acids (pooled
269 from membrane lipids and TAGs) since this would require *de novo* fatty acid synthesis (37).
270 We measured the individual classes of metabolite in the samples over the time course, and the
271 results are shown in Figure 4. In WT-12 cells, chlorophyll levels declined significantly as a
272 result of 0.1N treatment in both mixotrophic (TAP) and autotrophic (HSM) conditions ($P \leq$
273 0.001 between days 0 and 10), whereas there was no significant change in chlorophyll over
274 time in *sta6*. A similar significant decline in carotenoids was seen for WT-12 ($P < 0.01$)
275 though in *sta6* there was a slight increase in cells grown in 0.1N TAP and 0.1N HSM media.
276 In fact, the level of carotenoids in WT-12 and *sta6* cells grown in TAP was lower than those
277 grown in HSM from the start (Table 2, day zero), perhaps because the supply of organic
278 carbon suppresses biogenesis of the photosynthetic apparatus (12).
279 The polar lipid content is shown in Figure 4, middle panel. This decreased over time
280 in wild type cells grown in 0.1N HSM ($P = 0.02$) and when grown in TAP the decline was
281 relative to the increase in polar lipids in cells grown in 1N TAP ($P = 0.028$ at day 10). In
282 contrast, in *sta6* cells grown in HSM there was no change in polar lipids, but a substantial
283 decrease was observed in TAP medium ($P = 0.04$ from day 0 to day 10), despite little or no
284 change in chlorophyll.

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285 To determine if there were *de novo* synthesis of fatty acids under these conditions, we
286 then estimated the total fatty acids – ie the sum of fatty acids in TAGs + polar lipids + free
287 fatty acids – by transesterification of the lipid fraction to form fatty acid methyl esters
288 (FAMEs), which were then quantified by GC. In the wild type cells there was essentially no
289 difference in cells under any condition (Fig. 4, fourth panel), indicating that there was no net
290 fatty acid synthesis. In contrast, in *sta6* cells there was a 2-5 fold increase in FAMEs in 0.1N
291 conditions in both HSM ($P = 0.014$ from day 0 to 10) and TAP (though not statistically
292 significant due to the larger variances in 0.1N samples). The identifiable fatty acyl methyl
293 esters of the total FAME pool were predominantly 16:0 >18:3 >18:1 >18:2 > 18:0 (Fig. 5).
294 In WT-12, there were minor alterations in the composition of fatty acyl groups in cells grown
295 in 0.1N medium. In contrast for *sta6* cells, substantial changes in the profile of fatty acids
296 were seen, specifically there were spectacular increases in 16:0 in *sta6* cells grown in 0.1N
297 medium.
298 Lastly, we looked at the free fatty acid (FFA) pool (Fig. 4, bottom panel). In WT-12
299 cells, there was no alteration in FFAs under any condition, and although there was some
300 alteration in *sta6* grown in 0.1N HSM, it was not significant. In contrast, for the *sta6* cells
301 grown in 0.1N TAP there was a dramatic increase in these metabolites, with levels rising
302 from almost negligible to about 200 mg g^{-1} dry cell weight ($P = 0.015$ from day 0 to 10).
303 This increase in FFA, detected by GC-FID, was confirmed by two independent methods, thin
304 layer chromatography and GC-MS. Separation of the crude lipid extract by TLC, followed
305 by staining with 0.2% (w/v) 8-anilino-1-naphthalenesulphonic acid in methanol and
306 visualised under ultraviolet light showed accumulation in the region corresponding to FFAs
307 (by comparison with standards) for *sta6* in 0.1N TAP, but not in any other treatment (data not
308 shown). The identity was confirmed by eluting the FFAs from the TLC plate, esterification to
309 FAMEs, and then analysis by GC-MS. The underivatised FFAs from the total crude extract

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310 were also analysed by GC-MS. The mass profile of each peak was compared against
311 reference standards, NIST (http://www.nist.gov/srd/nist1a.cfm) and www.MassBank.jp mass
312 spectral libraries, and FFA identified as 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, and 18:4
313 (Supplementary spreadsheet file), the latter was not detected using GC-FID.
314 To establish whether the FFA accumulation was caused by degradation of
315 glycerolipids during extraction, a known lipase inhibitor, isopropanol, was added to
316 representative samples during the extraction procedure. Although the absolute amount of
317 FFAs was reduced somewhat, with a corresponding increase in polar lipids, there were still
318 over 100 mg FFA per g dry cell weight (~10% of cell dry weight) in *sta6* grown for 8 d in
319 0.1N TAP, 3 to 4 fold greater than the amounts measured in cells grown in 1N TAP.
320
321 ***Carbon (C) allocation and strain viability***
322 The allocation of carbon to each class of metabolite varied between the wild type and *sta6,*
323 and between the HSM and TAP growth media (Table 2, day 10). Under 1N conditions most
324 of the C in WT-12 and *sta6* cells was in polar lipids and chlorophyll (based on mg C g^{-1} dry
325 cell weight and mg C L^{-1} culture; Table 2, day zero). After 10 days of low N availability,
326 WT-12 cells allocated most of the C to starch, followed by TAG and polar lipids, whereas
327 *sta6* cells allocated most of their C to free fatty acids or polar lipids, followed by TAGs. The
328 amount of C allocated to TAGs was highest in *sta6* cells grown in TAP (122 mg C g^{-1} dry
329 weight) and the lowest was wild type cells grown in TAP (28 mg C g^{-1} dry weight).
330 However, when expressed as mg C per litre of culture, the culture that had the highest amount
331 of C allocated to TAG was wild type cells grown in HSM (133 mg C L^{-1}) and the lowest was
332 *sta6* cells grown in HSM (22 mg C L^{-1}), essentially due to the decreased growth of the *sta6*
333 cells growing under 0.1 N conditions over time (Fig. 6). To evaluate further the impact of
334 reduced growth in *sta6*, we grew the cells on HSM (no carbon source) or TAP (includes

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335 carbon source) agar plates in either constant light or diurnal (12 h light/ 12 h dark) regimes
336 for 7 days. As *sta6* cannot accumulate starch to use as a C source for respiration in the dark
337 period, we hypothesised that the growth of these cells would be severely affected when
338 grown under diurnal conditions, and this was indeed what was observed. Although *sta6* was
339 able to grow on TAP under both light regimes, it did not grow well on HSM and under
340 diurnal conditions it did not grow at all. In contrast WT-12 was able to grow, albeit more
341 slowly than in constant light (Fig 7).
342

343 **DISCUSSION**

344

345 ***Differences in carbon supply affect the initial rate of TAG accumulation***

346

347 In this study we have compared TAG production initiated by N limitation in wild type and
348 *sta6* cells of *C. reinhardtii* grown under autotrophic versus mixotrophic conditions. The
349 main hypothesis was that acetate, which could be used directly as a precursor for *de novo*
350 acetyl-CoA synthesis, would increase the initial rate of TAG production. Our results did
351 indeed show that the initial production of TAG was influenced by the type of C supply.
352 There was a nine-fold and 25-fold increase in TAG production in WT-12 and *sta6* cells
353 respectively grown in TAP, compared to cells grown in HSM (Fig. 2). However, this rate of
354 increase was not sustained, and the level of TAG reached a maximum after 2 or 4 days N
355 limitation in wild type and *sta6* cells, respectively. This indicates that the production of TAG
356 in cells provided with acetate is limited by C availability. The effect of C limitation in cells
357 grown in acetate has also been tested by Goodson et al. (38), where they observed an increase
358 in the number and size of oil bodies in cells given an "acetate-boost" after two days of N
359 starvation. Also, the fact that in our experiments WT-12 and *sta6* cells in autotrophic

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360 medium with a constant supply of CO_2 gradually increased their amount of TAG over time
361 implies that continued TAG production is related to C availability in the growth medium.
362 This is in line with Fan et. al. (4) who also concluded that C availability is a key factor in the
363 control of C partitioning between starch and TAG in *C. reinhardtii*, with TAG production
364 lagging behind starch production.

365 Interestingly, the trends in the increase in TAG concentrations in our study were
366 mirrored in the starch concentrations in N limited wild type cells, in that the initial rate of
367 starch accumulation in cells grown in TAP was faster than the starch accumulation in cells
368 grown in HSM, but the relationship between cellular content of the two storage molecules
369 was linear up to a maximum of ~500 mg starch.g^{-1} d.wt (Fig.3). Although Siaut et al. (7)
370 suggested that TAG and starch concentrations are not correlated, this may be the situation
371 once the maximum concentration of starch is achieved in the cell (after four days growth in
372 N-depleted TAP medium), rather than during the initial accumulation period.
373

374 ***There is less cellular autophagy of polar lipids in wild type cells supplied with acetate, but***
375 ***not in* sta6**
376

377 We also expected to observe *less* cellular autophagy of the structural lipids in thylakoid
378 membranes in cells supplied with acetate, as the rate of *de novo* synthesis of fatty acyl-ACPs
379 would be sufficient to supply fatty acids for TAG production, especially in *sta6* cells where
380 TAG production is greater than wild type cells. Metabolite changes (decrease in polar lipids
381 and pigments, no change in the total and individual fatty acids) indicated that TAG
382 production was the result of autophagy in WT-12 cells grown in HSM, and the level of
383 autophagy was less (in terms of the decrease in polar lipid concentrations) in cells grown in
384 TAP. However, this was not observed in *sta6* cells in HSM, since there was no change in

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385 polar lipid or chlorophyll, and the total fatty acids increased, with an alteration in profile
386 indicating *de novo* fatty acid synthesis. This indicates that the TAG production was mainly
387 from *de novo* synthesis of fatty acids rather than autophagy of cellular membranes (17, 18,
388 37).

389 Interestingly, the metabolic response in *sta6* cells grown in 0.1N TAP did indicate
390 some autophagy, since there was a decrease in polar lipids. This mix of autophagy and *de*
391 *novo* synthesis of fatty acids could explain why there was a higher concentration of TAG in
392 *sta6* cells grown in TAP than those grown in HSM. Our observation differs from that
393 described by Li et al. (11), who showed comparable concentrations of TAG production in a
394 starchless mutant grown in N deficient HSM and TAP. However, their result was based on
395 only two days of N-depletion. They also suggested that the accumulation of TAG was
396 predominantly from fatty acids synthesised *de novo* from photosynthetically fixed C, rather
397 than acetate, as the production of TAG in the starchless cells was severely reduced when
398 grown in TAP with no light.

399 The difference in the carotenoid levels between the wild type and *sta6* cells might also
400 explain the strain-specific autophagous responses to low N availability. Recent data by
401 Perez-Perez et al. (35) demonstrated that a decrease in carotenoids can trigger autophagous
402 processes in wild type *C. reinhardtii*. This correlation was also observed in our study where
403 wild type cells, with a reduction in carotenoids caused by N limitation, displayed
404 autophagous responses whereas the *sta6* cells with unaltered levels of carotenoids did not.

405 The fatty acid content in wild type and *sta6* cells grown in low N TAP was
406 comparable to that measured by James et al. (18) who reported 116 mg FAME g^{-1} dry cell
407 weight and 649 mg FAME g^{-1} dry cell weight in wild type (cc-124) and starchless (BAF-J5)
408 mutants of *C. reinhardtii*, respectively. However, unlike the studies by James et al. (18, 39)
409 we were unable to detect 16:3 and 16:4 fatty acids. They, alongside Msanne et al. (6), also

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410 reported that the major fatty acids were C16 and C18. This higher concentration of total fatty
411 acids, measured as FAMEs, in *sta6* cells compared to wild type, is partly due to the
412 substantial increase in the FFA component of the former. In the wild type cells, excess
413 photosynthate can be stored as starch, but this is not possible in *sta6* cell so the C is stored as
414 TAG, but it would appear that under mixotrophic conditions, there is further derailing of
415 metabolism leading to a substantial increase in FFA. This observation is significant, as the
416 increased availability of FFA could be exploited for further metabolic engineering
417 applications. Although total FAMEs have been reported to be higher in *sta6* cells when
418 compared to wild type cells (12), to our knowledge, this is the first report of the measurement
419 of FFA.
420
421 ***Increased TAG production per cell does not relate to increased TAG production per culture***
422
423 An understanding of the resources required for optimal TAG production in algal cells is of
424 paramount importance to biofuel industries, where culturing and fertilisers have the main
425 energy and economic demands in life cycle analysis models (1, 22, 40). Our research has
426 shown that mixotrophic growth increases the initial rate of TAG production per unit dry
427 weight. However, when expressed as mg C per L of culture, the most TAG was produced by
428 the wild type grown autotrophically (133 mg C per L culture). The main advantage that *sta6*
429 has over the wild type strains is that TAG is produced in the first two days of N limitation
430 when grown in TAP (23.6 mg TAG L^{-1} day^{-1}), whereas the maximum TAG productivity for
431 wild type cell was during day 8 and 10 when grown in HSM (34.3 mg TAG L^{-1} day^{-1}). Our
432 figures for wild type and *sta6,* although slightly lower than those quoted by Li et al. for *sta6*
433 (50 mg TAG L^{-1} day^{-1}) (10), are within the range of TAG productivity values quoted for
434 many algal species by Griffiths and Harrison (8). The reason for the difference in

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productivity between WT-12 and *sta6* was essentially because of the decreased biomass production of the *sta6* cells over time when grown in low N medium (Fig. 6), as previously already by James et al. (39) and Li et al (10). Moreover, *sta6* was not able to survive in diurnal conditions (Fig. 7), a phenotype also described in starch-less mutants of Arabidopsis (41), presumably because the lack of starch stores prevented respiration during the dark period. This novel and previous unreported observation would prevent cultivation of *sta6* in outdoor conditions, which would be most likely to prevail in an industrial context.

Finally, it should be emphasised that significant TAG production is only triggered when subjected to low N availability, and not when the C/N balance was altered by the inclusion of fixed carbon as acetate – since little TAG is produced in 1N TAP. This has been observed in other algal species (8, 42). Moreover, higher productivity over a sustained period was observed under autotrophic conditions, compared to the addition of fixed carbon (Table 1). Again, supply of gaseous CO_2 is likely to be more economically sustainable than the need to supply an exogenous organic carbon source.

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610 FIG. 1. Overlay neutral lipid (green fluorescence), chlorophyll (red fluorescence) and bright
611 field confocal microscopy images of *Chlamydomonas reinhardtii* (WT-12) cells grown in
612 normal (1 x Nitrogen) or low ($1/10^{th}$ Nitrogen) Sueoka's HSM + CO_2 growth media at day
613 two and day 10.

614

615 FIG. 2. Accumulation of total triacylglycerides and starch (mg metabolite per gram dry cell
616 weight) of *Chlamydomonas reinhardtii* (WT-12 or *sta6*) grown in either normal nitrogen
617 (1N) or low nitrogen (0.1 N) HSM or TAP media for 10 days. Data are mean and variance of
618 2-4 replicates.

619

620 FIG. 3. Correlation between total triacylglycerides and starch (mg metabolite per gram dry
621 cell weight) of *C. reinhardtii* (WT-12) grown in low nitrogen (0.1 N) HSM or TAP media.
622 Each point represents mean TAG and starch amounts from day 0 to day 10, shown
623 individually in Figure 2. Lines were fitted manually. Data are mean and variance of 2-4
624 replicates.

625

626 FIG. 4. Concentration of total chlorophyll, carotenoids, polar lipids, free fatty acids and total
627 FAME (mg metabolite per gram dry cell weight) of *Chlamydomonas reinhardtii* (WT-12 or
628 *sta6*) grown in either normal nitrogen (1N) or low nitrogen (0.1 N) HSM or TAP media for
629 10 days. Data are mean and variance of 2-4 replicates.

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633 FIG. 5. Concentration of total fatty acyl methyl esters (acid catalysed) of *Chlamydomonas*
634 *reinhardtii* (Wild type 12 or *sta6*) grown in either normal nitrogen (1N) or low nitrogen (0.1
635 N) HSM or TAP media for 10 days. Mean value for 16:0 in *sta6* 0.1 N TAP is 160 mg g dry
636 weight. Data are mean and variance of 2 replicates. Positions of unsaturations are given in
637 parenthesis.

638

639 FIG. 6. Biomass accumulation of *Chlamydomonas reinhardtii* (WT-12 or *sta6*) cultures
640 grown in either normal nitrogen (1N) or low nitrogen (0.1 N) HSM or TAP media for 10 days
641 expressed as dry biomass and optical density (absorbance at 750 nm). Data are mean and
642 variance of 2 replicates.

643

644 FIG. 7. Growth of *Chlamydomonas reinhardtii* (WT-12 or *sta6*) colonies grown on either 2%
645 HSM or 2% TAP agar for 7 days at constant or diurnal (12 light/12 dark hours) light. Each
646 colony originated from 5 µL aliquots of cultures that were normalised to ~9400 cells per 5
647 µL. Colony sizes were between 2-4 mm diameter.

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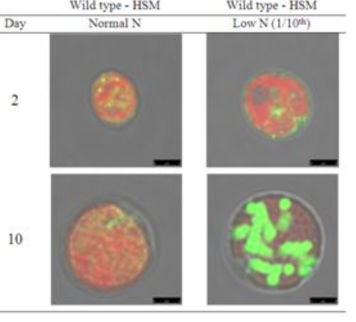

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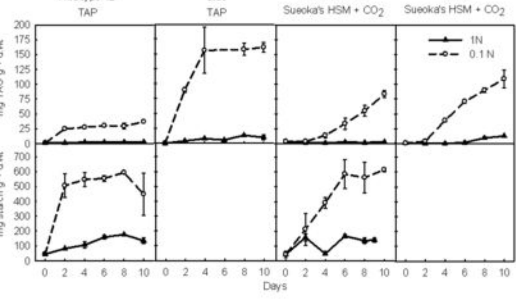

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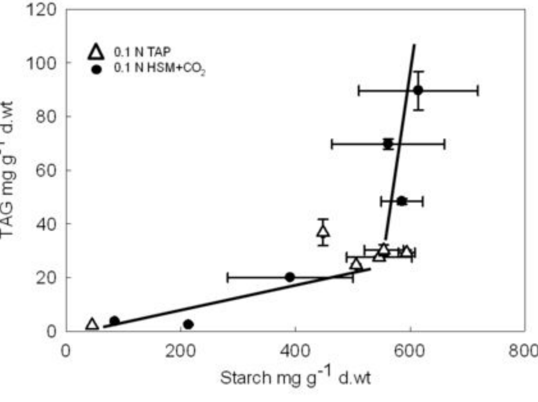

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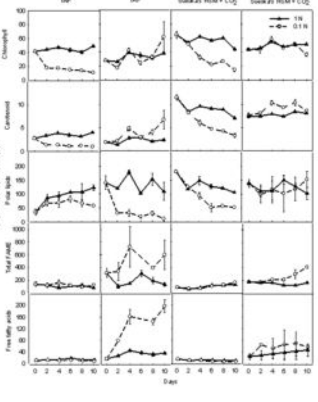

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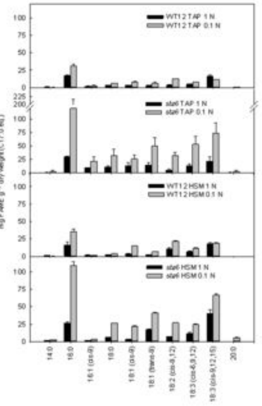

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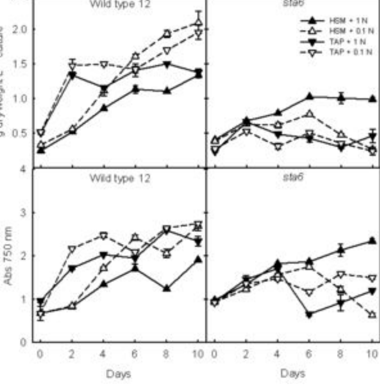

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Strain	TAP		HSM	
	Constant light	Diurnal light	Constant light	Diurnal light
Wild type-12				
sta6				

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TABLE 1: Productivity of triacylglycerides in *Chlamydomonas reinhardtii* (wild type 12 or *sta6*) grown in low nitrogen (0.1 N) Sueoka's High Salt Medium (HSM) or Tris-Acetate-Phosphate (TAP) media for 10 days. The daily rate of TAG production was calculated as the TAG level subtracted from the TAG level two days prior, divided by two. * = data between day 4 and 8. Expressed as mg g^{-1} dry cell weight day^{-1} or mg L^{-1} day^{-1}.

	mg TAG g dry weight^{-1} day^{-1}				**mg TAG L^{-1} day^{-1}**			
	TAP		**HSM**		**TAP**		**HSM**	
Days at 0.1 NH_4Cl	WT-12	*sta6*	WT-12	*sta6*	WT-12	*sta6*	WT-12	*sta6*
0 - 2	11.7	44.7	0.0	1.5	17.9	23.6	0.4	1.0
2 - 4	1.4	33.1	5.0	17.6	2.5	0.5	6.6	10.8
4 - 6	1.3	0.4*	10.0	15.9	0.8	1.8*	19.4	15.4
6 - 8	0	0.4*	10.7	9.5	3.4	1.8*	26.2	0.0
8 - 10	3.8	1.7	14.3	9.6	11.1	0.0	34.3	0.0

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TABLE 2: Allocation of carbon to different classes of metabolites in *Chlamydomonas reinhardtii* wild type 12 or *sta6* grown in low (0.1) nitrogen containing Sueoka's HSM + CO_2 or TAP growth media at day zero and day 10. The mean amount of carbon per class was based on the molecular formula of glyceryl tripalmitate (TAG), monogalactosyl diglyceride (polar), heptadecanoic acid (free fatty acids and FAME) (these were the commercial standards used for quantification via Gas Chromatograhy), chlorophyll *a* and β-carotene.

				DAY ZERO 0.1N (mg C per g dry cell weight)				
	medium	TAG	Starch	Chlorophyll	Carotenoid	Polar lipids	Free fatty acids	FAME
Wild type 12	TAP	1	20	31	3	26	8	105
sta6	TAP	0	0	22	2	98	12	235
Wild type 12	HSM	3	20	48	12	126	11	66
sta6	HSM	1	0	32	7	97	18	136
				DAY ZERO 0.1N (mg C per L culture)				
Wild type 12	TAP	1	13	20	2	16	5	67
sta6	TAP	0	0	6	1	30	4	73
Wild type 12	HSM	1	6	16	4	42	4	22
sta6	HSM	0	0	12	3	37	7	52

				DAY TEN 0.1N (mg C per g dry cell weight)				
	medium	TAG	Starch	Chlorophyll	Carotenoid	Polar lipids	Free fatty acids	FAME
Wild type 12	TAP	28	199	8	1	41	7	92
sta6	TAP	122	0	46	7	7	149	453
Wild type 12	HSM	63	273	11	3	37	5	124
sta6	HSM	83	0	27	8	107	43	309
				DAY TEN 0.1N (mg C per L culture)				
Wild type 12	TAP	55	390	16	2	81	13	181
sta6	TAP	30	0	11	2	2	36	110
Wild type 12	HSM	133	573	23	7	78	9	260
sta6	HSM	22	0	7	2	29	12	83