Integrated systems biology and imaging of the smallest free-living eukaryote *Ostreococcus tauri*

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Abstract:

Ostreococcus tauri is an ancient phototrophic microalgae that possesses favorable genetic and cellular characteristics for reductionist studies probing biosystem design and dynamics. Here multimodal bioimaging and multi-omics techniques were combined to interrogate *O. tauri* cellular changes in response to variations in bioavailable nitrogen and carbon ratios. Confocal microscopy, stimulated Raman scattering, and cryo-soft x-ray tomography revealed whole cell ultrastructural dynamics and composition while proteomic and lipidomic profiling captured changes at the molecular and macromolecular scale.

Despite several energy dense long-chain triacylglycerol lipids showing more than 40-fold higher abundance under N deprivation, only a few proteins directly associated with lipid biogenesis showed significant expression changes. However, the entire pathway for starch granule biosynthesis was highly upregulated suggesting much of the cellular energy is preferentially directed towards starch over lipid accumulation. Additionally, three of the five most downregulated and five of the ten most upregulated proteins during severe nitrogen depletion were unnamed protein products that warrant additional biochemical analysis and functional annotation to control carbon transformation dynamics in this smallest eukaryote.

Keywords:

systems biology, proteomics, lipidomics, biofuel, microalgae, photosynthesis, oleaginous, triacylglycerol, lipid, starch, carbon transformation, lipid droplet, nitrogen scavenging, soft x-ray tomography, stimulated raman scattering microscopy, fluorescence microscopy

Introduction

Microalgae are ubiquitous in oceans and maintain a major carbon sink in the complex world-wide ecosystem. Spanning more than one-billion years of evolution, microalgae are

26 phylogenetically diverse and exhibit varying cellular phenotypes that naturally produce high
27 value metabolites, proteins, carbohydrates and energy dense lipids that can be exploited for a
28 wide array of industrial applications [1-3]. Due to their high photosynthetic efficiency for energy
29 conversion, and minimal growth requirements consisting of sustainable resources such as
30 marine or brackish media, light, CO_2 and trace vitamins, microalgae are prime bioproduction
31 platforms [4,5].

32 Triacylglycerol (TAG) lipids are significantly enhanced when microalgae are subjected to
33 cellular stressors such as light, temperature, and nutrient deprivation [6]. TAG lipids possess
34 nonpolar character and are stored in anhydrous, high-density organelles called lipid bodies,
35 which are desirable for industrial lipid feedstock applications [7]. For other oleaginous algae, TAG
36 production can be triggered by nutrient deprivation of iron, sulfur, nitrogen, phosphate, or silicon
37 [6]. In most eukaryotes, combinatorial reduction of these nutrients results in altered levels of
38 growth-associated structural lipids (phospholipids) and energy storage lipids (TAG) products [8,9].
39 Unfortunately, in most cases starvation or deprivation can be detrimental to cell viability and
40 overall growth capacity thereby limiting cell biomass yields needed for viable lipid feedstock
41 industrial applications [10]. While several reports have shown that supplementing additional C
42 sources when combined with N depletion for certain organisms can yield increased growth and
43 lipid accumulation rates compared to strict starvation[2,11,12], a detailed understanding of the
44 interplay between carbon and nitrogen bioavailability is needed to advance bioproduction
45 applications.

46 Studying phototrophic metabolism in primitive species, such as the prasinophyte
47 *Ostreococcus tauri* can provide convenient opportunities to define minimal and critical metabolic
48 pathways for C transformation[13, 14]. *O. tauri* is the smallest known free-living eukaryote (~0.8μm
49 in thickness), lacks a cell wall, and thrives in varying photic, toxic and thermal ecosystems[15,16]. It
50 also has a highly condensed genome with only ~8,000 genes[17] so most reactions are governed

51 by a single enzyme (rather than multiple duplicating function enzymes) which simplifies
52 engineering requirements and interpretation. For example, the canonical model green alga
53 *Chlamydomonas* has 4 copies of Acetyl-CoA Carboxylase (E.C. 6.4.1.2) whereas *Ostreococcus*
54 has only 1 enzyme of that class. In addition, a recent study[18] reported the genetic diversity
55 associated with large phenotypic differences between *Ostreococcus* strains highlighting the
56 uncharted abundance of genetic biodiversity. These characteristics make *O. tauri* a potential
57 candidate for future industrial applications. However, considerable biodesign efforts will likely be
58 needed to develop an efficient cell factory for controlled and cost-effective bioproduction of lipid
59 feedstocks or other high value metabolites. Here, an integrated analysis of bioimaging,
60 proteomic, and lipidomic characterization was used to investigate *O. tauri* cellular response to
61 varying C:N ratios. Our results provide additional understanding of C storage and energy
62 transformation pathways within the microalgae *O. tauri* and identify new proteins to target for
63 future engineering efforts.
64
65 **Results**
66 Other than the bioavailable C:N ratio, all other experimental parameters (e.g.,
67 temperature, duration, diurnal cycling, light fluence, etc.) were kept constant throughout this
68 study. The four different media conditions used herein are designated as K6CN, K2CN, K2C
69 and K6C which are listed in decreasing C:N ratio. The normal growth media for *O. tauri* is Keller
70 Media (referenced herein as K2CN media) and contains ~2.5mM bicarbonate and 0.9mM total
71 nitrogen. To streamline interpretation, bicarbonate was kept as the sole media carbon source.
72 For K6CN media the nitrogen content was kept equivalent, but the bicarbonate increased to
73 6mM providing elevated carbon but normal nitrogen conditions. The use of 6mM bicarbonate as
74 the elevated carbon set point was chosen following an initial growth screen of *O. tauri* in K
75 media supplemented with various levels of bicarbonate from 0-10mM where 6mM showed

76 highest growth response with healthy chlorophyll ratios per cell. The final two media conditions
77 K2C and K6C share the same composition as K2CN and K6CN but are depleted of all nitrogen
78 sources. Thus all subsequent experiments consisted of 4 conditions of decreasing C:N
79 bioavailable ratios from elevated carbon with normal nitrogen (K6CN), to normal carbon with
80 normal nitrogen (K2CN), to normal carbon with no nitrogen (K2C), and elevated carbon with no
81 nitrogen (K6C).

82 Diurnal cycling with 12:12 hour light:dark cycles was used to synchronize cellular
83 division and growth. Cell growth was monitored by absorbance at 750nm (measure of
84 particulates) and 680nm (measure of chlorophyll a) for up to 144 hours (Fig. 1B). Decreases in
85 absorbance at 680nm were typical of K2C and K6C cell cultures relative to K2CN and K6CN.
86 Absorbance at 750nm exhibited similar decreases for N deprived cultures versus N replete.
87 However, when comparing normal C to excess C for either N replete or N deprived conditions,
88 cultures with excess C consistently displayed higher A680 and A750 values. Confocal
89 fluorescence microscopy was used to compare phenotypes for a couple dozen cells from each
90 condition whereas fluorescence activated cell sorting (FACS) allowed quantitative analysis of
91 larger population dynamics every 24 hours. In both case, dramatic increases of neutral lipid (NL)
92 content were detected for K2C and K6C conditions (Fig. 1 C–F). Interestingly K6CN (Fig. 1C)
93 cell cultures only exhibited subtle differences between NL and some increases in phospholipid
94 (PL) intensity indicating some photosynthetic lipid metabolism difference to K2CN (Fig. 1D).
95 Since confocal microscopy can only track dynamics for fluorescently labelled components,
96 label-free cell ultrastructure and composition changes were also evaluated.

97 Cryogenic soft x-ray nanotomography (CSXT) was performed on 72-hour cell cultures to
98 increase resolution and highlight native cellular features of intracellular lipid accumulation (Fig.
99 1E). Dark subcellular features, assigned as vacuoles due to their linear absorption coefficient
100 (LAC), were observed in the intracellular cytosolic space of cells in K6CN and K2CN culture

101 conditions, but no vacuoles were observed for K2C and K6C cultured cells. The vacuoles were
102 much larger in K6CN compared to K2CN conditions. Based on studies conducted in other algae
103 and plants starch is likely present in the chloroplast[17]. However, intracellular starch was
104 undetected in *O. tauri* cells via X-ray nanotomography likely due to the density appearing similar
105 to or masked by the surrounding tissues with a similar LAC - possibly due to its small cell size or
106 chloroplast packing density. Therefore, stimulated Raman scattering (SRS) microscopy was
107 used as a second label-free imaging method to identify relative abundance of intracellular starch
108 accumulation. Interestingly, starch was detected in the chloroplast for all tested conditions.
109 However, the starch seen for K2C was minimal (Supplemental Figure 1). Considering that K6C
110 represents an even more deprived nitrogen to carbon ratio compared to K2C, it was anticipated
111 that K6C would show the lowest levels of starch since both conditions also show significant lipid
112 accumulation. However, the increase in starch content for K6C suggests that these cells still
113 have abundant carbon available for transformation into carbohydrates, and potential future
114 engineering efforts could focus on knocking out genes associated with starch accumulation to
115 divert this excess energy toward lipid feedstock production instead.

116 The confocal imaging also happened to capture a possible lipid droplet secretion event.
117 During live-cell imaging, a single cell was captured over a few minutes showing the formation of
118 a cellular bleb containing a single lipid droplet that was released in subsequent scans
119 (Supplemental Figure 2). We have previously reported that this organism does not appear to
120 have any canonical proteins associated with lipid droplet secretion from other organisms[19]. That
121 study also captured static images showing what was described as blebbing intermediates. The
122 current image series reported here is the first case in which the process was observed live
123 thereby lending additional support to the theory that *O. tauri* is capable of lipid droplet secretion
124 although the detailed mechanism remains elusive.

125 Dramatic changes observed for lipid staining profiles prompted the exploration of
126 underlying global proteomic expression profiles for each experimental culture condition.
127 Cultures were harvested at 24- and 48-hour time points for LC-MS/MS proteomics to obtain a
128 measure of global proteomic expression. These time-points were chosen for comparison since
129 they showed the biggest relative 24-hour changes via FACS and confocal analysis. The
130 proteomics data was mapped into individual metabolic groups (synthesis, degradation, energy,
131 other and non-metabolic pathways) to interpret relative changes of cells cultured in K6CN,
132 K2CN, K2C and K6C conditions for each metabolic pathway (Fig. 2). Of particular note was the
133 increased changes in abundance for both K2C and K6C in the carbohydrate synthesis pathway
134 compared to K2CN and K6CN. However, very few proteins within the fatty acid (FA)/lipid
135 synthesis pathways showed significant, if any, change in abundance despite bioimaging
136 observations of cellular lipid increases up to 60% the volume of cells. For example, while DGAT,
137 the canonical enzyme representing the last committed step of TAG synthesis was detected with
138 global proteomics, it's abundance remained effectively equivalent across all 4 sample
139 conditions.
140 High C to N ratio of K6C displayed consistently higher proteomic responses compared to
141 K2C for upregulated proteins related to C storage. The complete starch pathway was detected
142 with ascending upregulation from alpha amylase to granule-bound starch synthetase (GBSSI)
143 for N depletion conditions. GBSSI was the third highest upregulated protein overall highlighting
144 the reliance on carbon storage under these conditions. Previous studies have found similar
145 results for GBSSI[20]. N deprivation also caused downregulation of proteins involved in N
146 acquisition, such as nitrate transporters, nitrate reductase with concomitant upregulation of N
147 scavenging proteins for glutamine, asparagine, and urea. Much of the downregulated proteins
148 detected were ribosome based or were proteins localized to the chloroplast.

149 Overall 471 unnamed protein products were found to be upregulated or downregulated
150 with several exhibiting some of the most extreme abundance changes (Fig. 4). N deplete
151 conditions K2C and K6C had similar distributions of upregulated and downregulated protein
152 trends, with more increased upregulation of proteins for excess carbon cultures. For K6C, 5 of
153 the 10 most upregulated and 3 of the 10 most downregulated were UPP (Fig. 3). Interesting,
154 several of these proteins show inversion of abundance for replete versus depleted N conditions.
155 XP_003075209, XP_003081059, XP_003078347 all exhibit significant upregulation under K6CN
156 and K2CN conditions at 48 hours compared to K2CN at 24 hours, however, these proteins show
157 significant downregulation for K2C and K6C conditions at 48 hours. The biggest change was
158 seen for XP_003078347 which was had a log2 value change of -0.54 for K6CN but a value of
159 1.96 for K6C. Similarly, other unnamed proteins XP_003084215, XP_003082140 and
160 XP_003082699 were all downregulated for K6CN and K2CN at 48 hours but upregulated for
161 K2C and K6C. Clearly these unnamed proteins are dramatically affected by the bioavailable
162 ratio of C and N and represent interesting targets for future in-depth functional annotation.
163 Conducting a BLAST alignment analysis on the UPP identified in these runs resolved a
164 predicted membrane protein (XP_003080099) upregulated for N depleted conditions K2C and
165 K6C, which was identified as having a domain with homology to TMEM14, an uncharacterized
166 superfamily believed to be involved in membrane transport of lipids in higher eukaryotes. This
167 protein could possibly play a role in the lipid droplet secretion from *O. tauri*. Other potentially
168 interesting unknown protein products were also uncovered during BLAST alignments with two
169 notable probable identifications: A putative Acyl-CoA N-acyltransferase (XP_003084085.1)
170 slightly upregulated for K2CN and K2C but not K6CN or K6C; and a putative Zinc finger
171 (XP_003080741.1) slightly downregulated for K6CN, K6C, and K2C conditions, which have
172 been known to participate in a number of eukaryotic cellular mechanisms including lipid binding.
173 Additional proteins such as sarcosine-dimethyltransferase (SDMT), an enzyme found in the

174 betaine biosynthesis pathway in other algae and higher plants related to osmoprotection during
175 cellular stress[21,22], were found to be elevated only for K2C and K6C conditions, which may
176 reinforce cellular stability during N stress and lipid accumulation.

177 Despite observing limited changes in the proteome related to lipid metabolism, the visual
178 confocal and FACS analysis provided ample evidence of significant lipid accumulation that
179 warranted further characterization to understand cellular lipid composition and relative
180 abundance. Thus, the same K6CN, K2CN, K2C, and K6C cultures were surveyed at the same
181 24- and 48-hour time-points using LC-MS/MS global lipidomics analysis. Quantitative lipid
182 profiles were collected for more than 280 lipids (Supplemental Figures 3 – 9). The N depletion
183 conditions (K2C and K6C) resulted in significant increases of energy dense TAG lipids with
184 more than 10 TAGs showing >40-fold increase in abundance already at 48-hours. The FACS
185 and confocal analysis showed that the NL content continues to accumulate well beyond 48
186 hours. In comparison, TAG lipid abundance was flat or decreased for K6CN and K2CN
187 conditioned cells, indicating that cells were not diverting C into lipid energy storage during
188 nutrient rich conditions although they were still accumulating starch as seen from SRS
189 (Supplemental Figure 1). The observed FA profiles provide additional support for recently
190 reported[23] unique long chain FAs despite lacking annotated enzymes known to synthesize
191 them, indicating that *O. tauri* is at the very least an intriguing oleaginous organism for lipid
192 feedstock development. In addition, the number of long chain FAs in TAGs were significantly
193 enhanced during K6C conditions, demonstrating this organism's capacity to uptake and
194 transform excess C into long chain energy dense lipids without genetic modification
195 (Supplemental Figures 7–9).

196 Changes in structural lipids were also detected (Supplemental Figures 3 – 6).
197 Thylakoidal membranes are composed of monogalactosyldiacylglycerol (MGDG) and
198 digalactosyldiacylglycerol (DGDG), which stabilize the thylakoid for maximal photosynthetic

199 efficiency [24]. In addition, algal thylakoid membranes contain abundant amounts of
200 sulphoquinovosyldiacylglycerol (SQDG) which contributes to increased stability in the
201 photosynthetic harvesting complexes and accommodates membrane protein associations
202 unique to microalgae [25]. Increased abundances for MGDG and 3- to 7-fold increases in DGDG
203 were detected for K2C and K6C conditions, with C16 chain lengths exhibiting the most
204 abundant and dramatic changes during N depletion. C16 chain length SQDG lipids were also
205 slightly upregulated for K6C, suggesting they may be adding stability to light harvesting
206 complexes or membrane protein expression unique to excess C exposure and N depletion.
207

208 **Discussion**

209 Linkages between N and C metabolism related to lipid biogenesis were interrogated for
210 the microalgae *Ostreococcus tauri*. Single cell and population imaging experiments combined
211 with global proteomic and lipidomic experiments on the same cultures demonstrated that even
212 the simplified cell architecture and genome of *O. tauri* displays complicated regulatory linkages
213 as a function of bioavailable carbon to nitrogen ratios. Cryogenic soft x-ray nanotomography
214 revealed distinct lipid droplet distributions of varying sizes confirming initial fluorescence
215 microscopy results. K2C conditioned cells had uniform sized lipid droplets whereas cells in K6C
216 conditions had lipid droplets of varying size including very large lipid droplets that swelled and
217 deformed the cells.

218 Numerous photosynthetic, structural, and energy storage fatty acid (FA)/lipids were
219 verified through LC–MS/MS lipidomic analysis that included detection of long chain TAGs, ideal
220 for lipid feedstocks. Proteomic data combined with physiological cell responses to varying C and
221 N revealed that although FA/lipid synthesis pathways had little proteomic changes,
222 carbohydrate synthesis proteomics was independently upregulated. Both ribulose 1,5-
223 biphosphate carboxylase/oxygenase large and small subunits (Rubisco) were slightly

224 downregulated for K2C and K6C relative to K2CN suggesting Rubisco expression in
225 *Ostreococcus* contains minimum concentrations of Rubisco to support normal growth as
226 reported for other organisms[26]. Restricting protein identifications to subsets related to
227 carbohydrate conversion, N scavenging, and energy regulation allowed for the simplification of
228 biological interpretations related to lipid feedstock optimization targets. The most upregulated
229 protein was a protein kinase involved in serine/threonine phosphorylation and was equally
230 upregulated for K2C, K6C, and K6CN conditions, which could possibly play a role in regulating
231 diurnal cycling or stress response since K6CN could also be seen as another N deprived state
232 relative to available C. GBSSI was the third most upregulated protein in all and was more
233 upregulated in K6C than K2C. Knock-down of GBSSI could potentially provide a redirection of
234 *O. tauri* metabolism to less starch accumulation and more lipid accumulation under K6C
235 conditions.
236 In addition to the proteins related to energy storage, increased abundances of proteins
237 related to lipid viability, lipid production, and osmotic shock were detected to varying degrees.
238 Finally, a surprising number of proteins of unknown function and identity were revealed to be
239 part of the 10 most significant proteomic increases and decreases overall and each is a
240 potential target for future engineering efforts. Furthermore, the wealth of undefined proteins
241 emphasizes the extent of unexplored opportunities related to N, C, and energy storage
242 pathways, and highlights the peculiar genetic diversity within natural populations of
243 *Ostreococcus* and possibly other primitive marine species.
244
245 **Methods:**
246 **<u>Strain maintenance, culture growth media, and nutrient starvation conditions</u>**
247 *O. tauri* cell cultures were obtained from the Roscoff Culture Collection (RCC745); strain name:
248 OTTH0595, which has been fully sequenced[17]. Cultures of RCC745 were grown in defined

249 Keller (K) media[27] with normal or depleted N and HCO^{3-}: K2CN contained normal N and C,
250 K6CN with normal N and 6mM HCO^{3-}, K2C with depleted N and 2.5mM HCO^{3-}, and K6C with
251 depleted N and 6mM HCO^{3-}. All Keller media-based culture conditions were prepared in fresh
252 artificial seawater (ASW) with defined amounts of nutrients analytically prepared fresh and
253 sterile filtered prior to each experiment. To monitor growth and lipid accumulation over time
254 absorbance at 680nm and 750nm was measured to obtain both values for chlorophyll content
255 and particulate matter, respectively, for each culture. Graphing the ratio of 750nm/680nm
256 provided a measure of chlorophyll functional efficiency as well as possible lipid particulates in
257 solution. Graphical analysis of each growth and starvation curve required minimal normalization
258 due to our consistent efforts in capturing cells during mid-log stages of growth. For growth and
259 lipid accumulation studies cells were grown initially in normal K media to approximately 0.03 at
260 OD680 then gently centrifuging cultures at 1200XG for 10 minutes in a swing bucket rotor,
261 washed with respective defined K media, and resuspending cells into defined K media at a
262 target 0.03 at OD680 and continued diurnal light entrainment for specified time courses in
263 sealable CytoOne non-treated cell culture flasks (USA Scientific, USA) with mixing of cultures
264 once per 24 hours. To prepare cell cultures for starvation surveys they were gently centrifuged
265 fresh cultures at 2200xG for 10 mins with swing bucket rotor centrifugation and washed cell
266 pellets once with defined K media of interest then suspended cells in defined media conditions
267 and continued diurnal light entrainment for specified time courses in sealable CytoOne non-
268 treated cell culture flasks (USA Scientific, USA) with mixing of cultures once per 24 hours.
269

270 **Fluorescence activated cell sorting analysis of intracellular lipid content**

271 *O. tauri* cells were cultured to mid log phase and gently centrifuged to concentration then
272 stained with Nile red lipid stain for exactly 10 mins before each experimental measurement on
273 the BD INFLUX flow cytometer (BD Biosciences, San Jose, CA, USA). FSC and SSC were

274 used to gate out any non-specific cellular debris. Specific gating in the range of known cell size
275 of *O. tauri* was used to determine the fluorescence from stained neutral lipid (488/542±13.5 nm),
276 phospholipid (561/615±12 nm), and natural chlorophyll autofluorescence (640/670±15 nm) for
277 defined populations of cells. Each individual FACS experiment was calibrated to 3.6 ide scatter
278 10 mins before running our sample measurements in defined media cultures. The fluorescence
279 intensity of neutral lipid and phospholipid fluorescence intensity at specific time points was
280 compared in scatter plots to demonstrate population dynamics for each sample condition. K2CN
281 at 24 hours was used as the baseline for normal conditions to detect changes due to varying
282 C:N bioavailability.
283
284 **Fluorescence and SRS confocal microscopy**
285 Confocal images were obtained on Zeiss LSM 710 (Carl Zeiss AG, Germany) confocal
286 microscope with a 100x oil immersion objective. An InTune Laser with 505nm and 535nm light
287 was used to maximize the separation of the triglyceride (585nm) and phospholipid (638nm)
288 emission peaks while diminishing crosstalk of the Nile Red Stained cells. In addition, chlorophyll
289 autofluorescence was excited with 405nm light and monitored the emission profile at 680nm.
290 Nile Red stained cells were immobilized on glass slides with poly-L-lysine and imaged
291 immediately with z-scan slicing of 0.43μm to survey whole cell fluorescence labeling distribution.
292 All fluorescence channels were set with identical gain and laser power settings to provide
293 relative levels of fluorescent intensity. No adjustments of contrast or gain were applied during
294 post processing. Microalgal cells were also imaged on a Leica DMi8 Confocal Microscope with
295 HyD Detector using a 40× 1.10 NA water immersion objective with 1× and 4× zoom to satisfy
296 Nyquist frequency. Auto-fluorescence of chlorophyll (red) was excited at 552 nm, and emissions
297 were monitored at 674–684 nm and then displayed over bright-field channel.
298

299 **Cryogenic Soft X-ray Tomography for Intracellular Distribution of Organelles**

300 Cell cultures were grown on-site at the Advanced Light Source (ALS) at Lawrence
301 Berkeley National Laboratory with a homemade 470nm light source with a measured intensity of
302 20μE. Cell cultures were incubated with 12-hour diurnal light at RT and harvested by
303 centrifugation at 48, 72 and 96-hour time points. Cells were gently centrifuged at 1000xG for
304 10mins to pellet. Pelleted cells had all but ~5μL of supernatant removed to remove a viscous
305 cell biomass. Wet cell biomass was inserted into 5μm micro capillaries and plunge frozen in cold
306 liquid propane. In some cases, 6μm polystyrene beads were added to cell suspensions prior to
307 centrifugation to minimize the impact of freezing on large lipid containing cells. Frozen
308 capillaries were stored in liquid nitrogen until imaging by the soft x-ray light source. Soft x-ray
309 data acquisition was carried out on beamline 2.1, a soft x-ray microscope in the National Center
310 for X-Ray Tomography (NCXT) located at the Advanced Light Source in Berkeley, California[28].
311 The microscope soft x-ray illumination was generated by a bend-magnet in the synchrotron
312 lattice and focused onto the specimen by a Fresnel Zone Plate (FZP) condenser. Specimen
313 illumination was order-sorted by a pinhole positioned just in front of the specimen. A second
314 zone plate, located downstream of the specimen, magnified and focused an image of the
315 specimen on a CCD detector. During data collection, the cells were maintained in a stream of
316 helium gas that had been cooled to liquid nitrogen temperatures. Each tomographic dataset
317 (i.e., 90 projection images spanning a range of 180°) was collected using Fresnel zone plate
318 based objective lens with a resolution of 50 nm. Exposure times for each projection image
319 ranged from 150 to 300 msec. The software suite AREC3D was used to align the projection
320 images calculate tomographic reconstructions[29].

321

322 **Lipid Extraction and Lipidomics Using Liquid Chromatography Tandem Mass**
323 **Spectrometry**

324 Each *O. tauri* culture was harvested at specific time points and ultimately spun down at
325 3000 x g for 10 min to form a pellet in chloroform compatible 2 mL Safe Seal microfuge tubes
326 (Sorenson Bioscience, Inc, Salt Lake City, UT). The supernatant was removed, and the wet
327 pellet was weighed for an estimate of biomass, then a mixture of 1 mL of methanol, 0.5 mL of
328 chloroform and 0.4 mL of nanopure water was added to each pellet. The pellets were vortexed
329 and sonicated for about 10 sec in a bath sonicator and the mixture was allowed to stand at room
330 temperature for 10 min. Then an additional 0.5 mL of chloroform and 0.5 mL of water were
331 added, and the mixtures were shaken vigorously into an emulsion. Each sample was
332 centrifuged at 10,000 x g for 10 min. The bottom chloroform layer was carefully removed from
333 the separated solvent layers using a Pasteur pipette and taking care not to take up any of the
334 protein disc or the top polar layer and was placed in a pre-weighed glass auto sampler vial. The
335 bottom lipid layer was dried down overnight under nitrogen and the vials with the total lipid
336 extract (TLEs) were weighed to obtain the total mass. 5μL of chloroform was added to each
337 dried sample and then the sample diluted to 50 μg/μL with methanol. The samples were then
338 stored at –20 °C until ready for mass spectrometric analysis.
339 Extracted lipids were dried down then reconstituted in methanol. The TLEs were
340 analyzed by LC-MS/MS using a Waters NanoAquity UPLC system interfaced with a Velos
341 Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) as outlined in Dautel et al.
342 2017[30]. LC-MS/MS raw data files were imported into the in-house developed software LIQUID[31]
343 for identification of lipid molecular species. Manual validation of the lipid identifications were
344 determined by examining the tandem mass spectra for diagnostic ion fragments along with
345 associated chain fragment information. In addition, the isotopic profile, extracted ion
346 chromatogram (XIC), and mass error of measured precursor ion were examined for lipid
347
348 **Cellular Protein Extraction and Digestion**

349 To the remaining protein/debris pellet obtained from the lipid extraction, 1 mL of ice cold
350 methanol was added 3 x to wash the majority of residual metabolites away from the pellet. A
351 Filter-Aided Sample Prep (FASP) protein extraction and trypsin digestion was then performed
352 using the FASP protein digestion kit (Expedeon, San Diego CA) using manufacturer's
353 suggested protocol (Erde J et al, 2014, J Proteome Res 13(4):1885-1895). Briefly, methanol
354 washed pellets were air dried for 2 hrs and were resuspended in solubilization buffer of 12 mM
355 deoxycholate, 12 mM N-lauroyl sarcosine with 10 mM TCEP, and 200 mM ammonium
356 bicarbonate, pH 8.0 at an approximate concentration of 13 μg/μL. 30 μL of this protein solution
357 was then mixed with 200 μL of urea sample solution (kit provided), the sample was centrifuged
358 on the kit provided spin filter at 14,000 x g for 15 min. Washes with urea and ammonium
359 bicarbonate along with trypsin digestion and alkylation with iodoacetamide were carried out as
360 the kit specifies. Peptides were then suspended in nanopure water and peptides were then
361 quantified using a BCA assay (Pierce, Rockford IL) with a bovine serum albumin standard.
362
363 **iTRAQ Peptide Labeling**
364 Peptides were labeled with 8-plex iTRAQ (AB Sciex, Redwood City, CA) reagents as
365 described below. 100 μg of each peptide sample was placed in a new tube and dried down.
366 Chanel designations are as follows: 43 μg of dissolution buffer (iTRAQ buffer kit) was added to
367 each sample, these being vortexed into solution and centrifuged briefly to draw sample to the
368 bottom of each tube. The iTRAQ reagent (30 μL) was diluted further with isopropanol (115 μL)
369 and this was then added to each sample. Each reaction was carried out at RT for 2 hrs, with 50
370 mM ammonium bicarbonate (200 μL) added to quench each reaction tube. After 1 hr, the
371 contents from all iTRAQ channel reactions were added to one tube and then the sample was
372 vortexed and dried down in a speed vac. The labeled peptides were cleaned up using C-18
373 SPE columns (SUPELCO Discovery) were then employed to remove the salts, using a 0.1%

374 TFA in nanopure water to wash the peptides and 80% acetonitrile, 0.1% TFA in water to elute
375 the peptides.
376
377 Offline Fractionation of Peptides and Preparation of Proteome Samples
378 400 μg of iTRAQ labeled peptides were separated using an off-line high pH (pH 10) reversed-
379 phase (RP) separation with a Waters XBridge C18 column (250 mm x 4.6 mm column
380 containing 5 μm particles and a 4.6 mm x 20 mm guard column) using an Agilent 1200 HPLC
381 System. The sample loaded onto the C18 column was washed for 15 min with Solvent A (10
382 mM ammonium formate, adjusted to pH 10 with ammonium hydroxide). The LC gradient started
383 with a linear increase of Solvent B (10 mM ammonium formate, pH 10, 90% acetonitrile in
384 water) to: 5% over 10 min, 45% Solvent B over 65 min, and then a linear increase to 100%
385 Solvent B over 15 min. Solvent B was held at 100% for 10 min, and then was changed to 100%
386 Solvent A, this being held for 20 min to recondition the column. The flow rate was 0.5 mL/min.
387 A total of 96 fractions were collected into a 96 well plate throughout the LC gradient. The high
388 pH RP fractions were then combined into 12 fractions using the concatenation strategy
389 previously reported[32]. Peptide fractions were dried down and re-suspended in nanopure water
390 at a concentration of 0.075 μg/ μL for mass spectrometry analysis using a Q Exactive HF Hybrid
391 Quadrupole-Orbitrap MS (Thermo Scientific) system as described below.
392
393 **Mass-Spectrometry Based Analysis of Samples**
394 All peptide samples were analyzed using an automated home-built constant flow nano
395 LC system (Agilent) coupled to an Q Exactive HF Hybrid Quadrupole-Orbitrap MS (Thermo
396 Fisher Scientific). Electrospray emitters were custom made using 150 μm o.d. x 20 μm o.d. x
397 20 μm i.d. chemically etched fused silica. An on-line 4-cm x 360 μm o.d. x 150 μm i.d. fused-
398 silica capillary analytical column (3 μm Jupiter C18) was used. Mobile phases consisted of

399 0.1% formic acid in water (A) and 0.1% formic acid acetonitrile (B) operated at 300 nL/min with
400 a gradient profile as follows (min:%B); 0:5, 2:8, 20:12, 75:35, 97:60, 100:85.
401
402 **Peptide Identification, Quantification and Analysis**
403 For peptide identification, MS/MS spectra were searched against a decoy O. tauri
404 database using the algorithm SEQUEST. An approach to correlate tandem mass spectral data
405 of peptides with amino acid sequences in a protein database[33]. Search parameters included: no
406 enzyme specificity for proteome data and trypsin enzyme specificity with a maximum of two
407 missed cleaves, ± 50 ppm precursor mass tolerance, ± 0.05 Da product mass tolerance, and
408 carbamidomethylation of cysteines and iTRAQ labeling of lysines and peptide N-termini as fixed
409 modifications. Allowed variable modifications were oxidation of methionine. MSGF+ spectra
410 probability values were also calculated for peptides identified from SEQUEST searches[34].
411 Measured mass accuracy and MSGF spectra probability were used to filter identified peptides to
412 <0.4% false discovery rate (FDR) at spectrum level and <1% FDR at the peptide level using the
413 decoy approach. iTRAQ reporter ions were extracted using the MASIC software[35] for fast
414 quantitation and flexible visualization of chromatographic profiles from detected LC-MS(/MS)
415 features with a 10-ppm mass tolerance for each expected iTRAQ reporter ion as determined
416 from each MS/MS spectrum.
417 Relative abundances of peptides were determined using iTRAQ reporter ion intensity
418 ratios from each MS/MS spectrum. Individual peptide intensity values were determined by
419 dividing the base peak intensity by the relative ratio associated with each reporter ion. All
420 peptide values were then transformed into log2 values for comparison between conditions.

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Competing Interests: We declare no competing interests.

Contributions

JEE devised and managed all experiments with input from CRS. CRS conducted confocal and Raman imaging experiments and coordinated integrated omics analysis. KKH and CDN performed lipid and proteomics sample prep. RM collected proteomics data that SP analyzed, and NK and WRC worked into the cell model. JEK performed lipidomic data collection and lipidomic analysis. Cryogenic soft X-ray sample preparation was conducted by JHC, RB and CRS with JHC and GM acquiring tilt series for subsequent reconstruction by AE. FACS analysis was conducted by WC. JEE and CRS wrote initial manuscript and all authors edited and approved final text.

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Main Figures

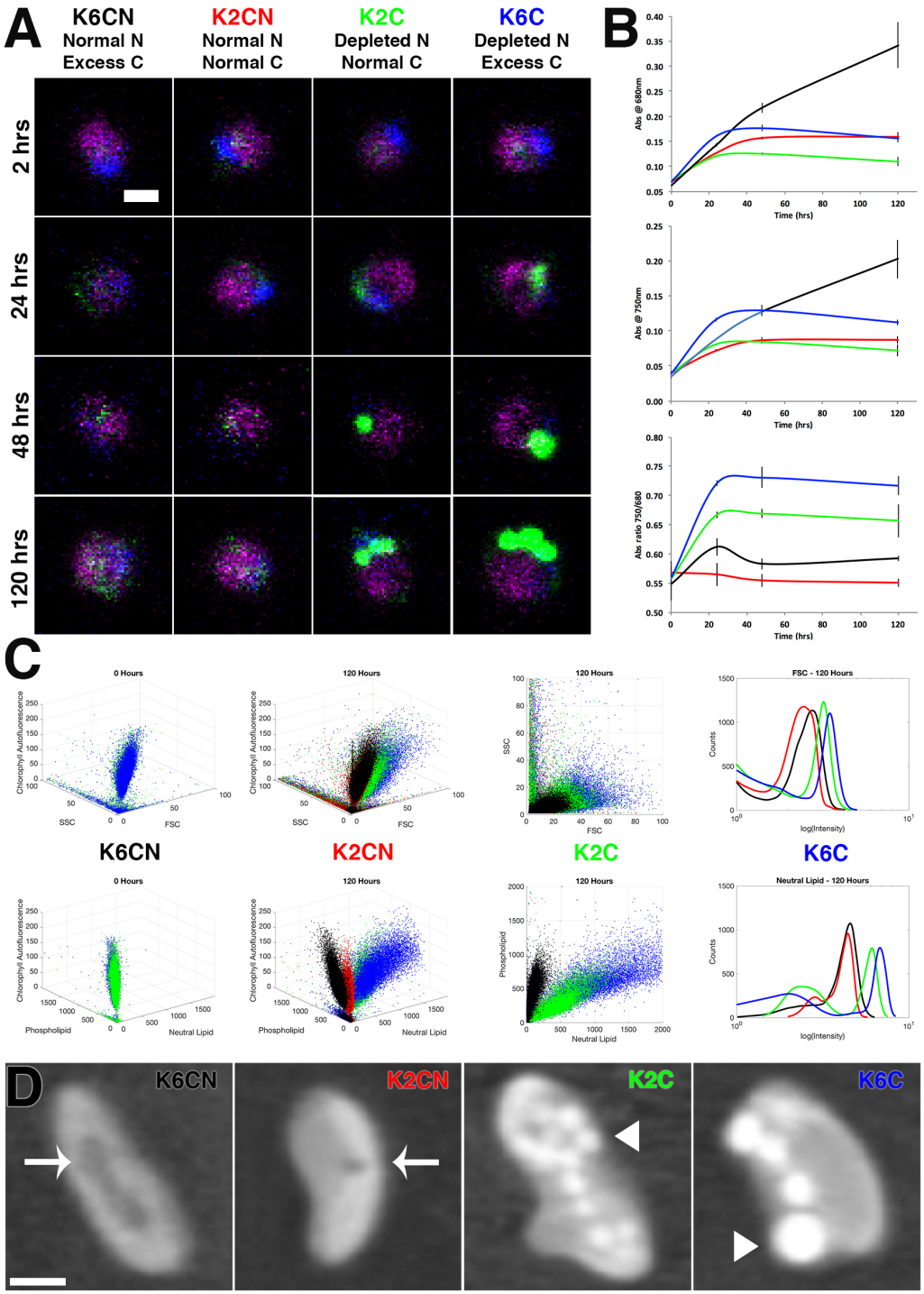


Figure 1 Single cell and population imaging and growth under varying C:N ratios
(A) Confocal microscopy of K6CN, K2CN, K2C, and K6C cultures to observe chlorophyll autofluorescence (magenta), nucleic acid fluorescent staining (blue), neutral lipid fluorescent staining (green) over time. Scale bar represents 1µm scale. (B) Corresponding growth plots for the same cultures monitored over time (error bars represent a replicate of 5) at 750nm and 680nm, and the ratio of the 750/680 as a measure of photosynthetic efficiency. (C) FACS time course study of same cultures comparing SSC (side-scattering) versus FSC (forward scattering) and chlorophyll autofluorescence in 3D plots at 0 hours and 120 hours, then 2D plot of SSC versus FSC at 120 hours, and histogram of FSC at 120 hours. (D) FACS time course study of same cultures comparing phospholipid versus neutral lipid and chlorophyll autofluorescence in 3D plots at 0 hours and 120 hours, then 2D plot of phospholipid versus neutral lipid at 120 hours, and histogram of neutral lipid at 120 hours. (E) Label-free cryogenic soft x-ray nanotomography images of cells cryogenically frozen in microcapillaries. Central slices (1µm scale bar)

display intracellular structures common to each culture condition. Chloroplasts and cytoplasm seen in all images. Vacuoles in K6CN and K2CN appear as dark objects indicated with white arrow. Lipid droplets appear as bright white circular objects indicated by white arrowhead.

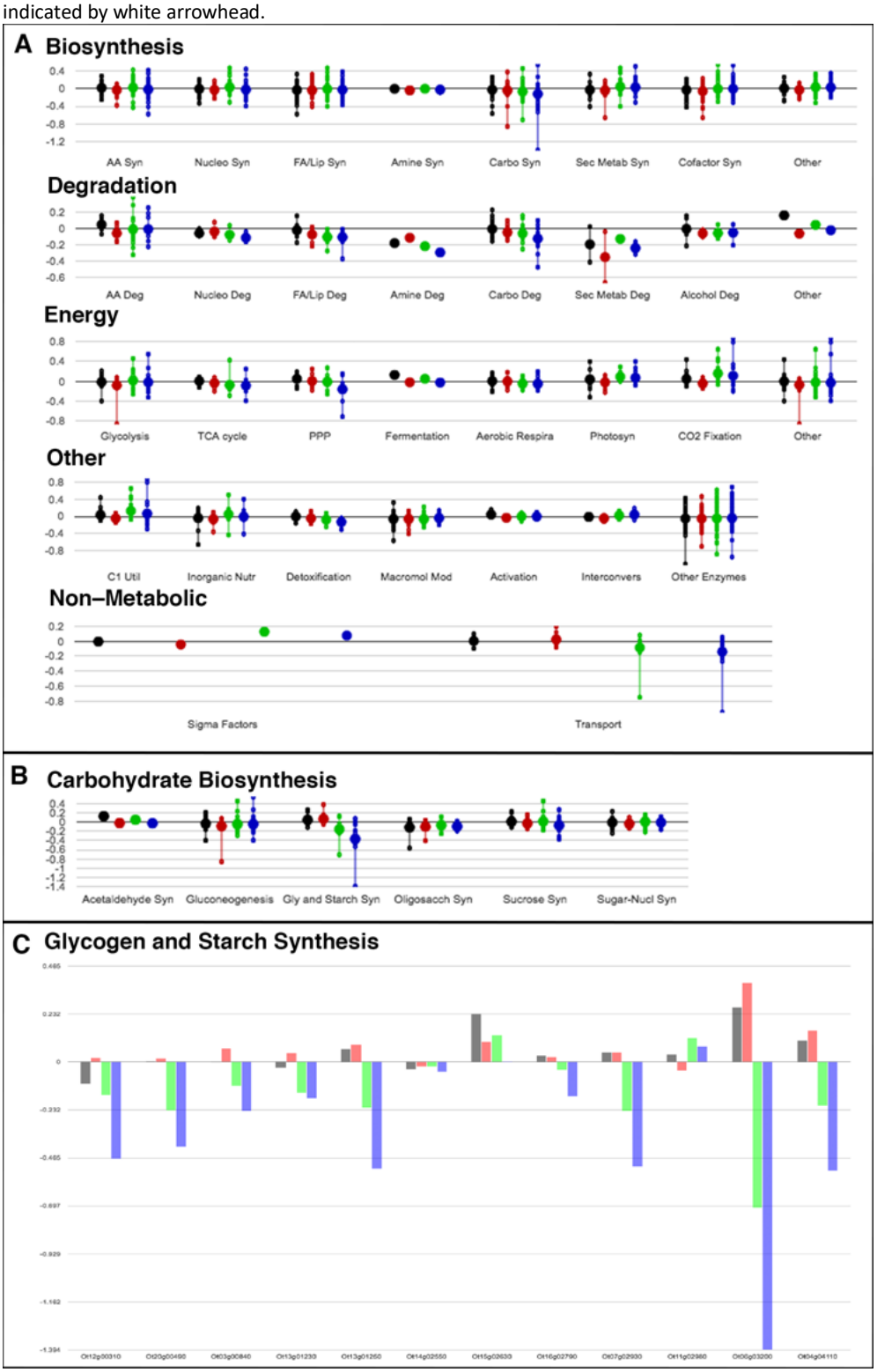


Figure 2 Metabolic pathway mapping of global proteomics

O. tauri cells cultured in K6CN (black), K2CN (red), K2C (green), K6C (blue) at 48 hours relative to cultures from K2CN at 24 hours were grouped and mapped by metabolic pathway(A), subpathway (B), and individual proteins (C). Each large circle represents

an average level of abundance on a log2 scale where negative values are increases in abundance relative to cell conditions K2CN at 24 hours. Smaller circles represent individual proteins in the respective metabolic pathway.

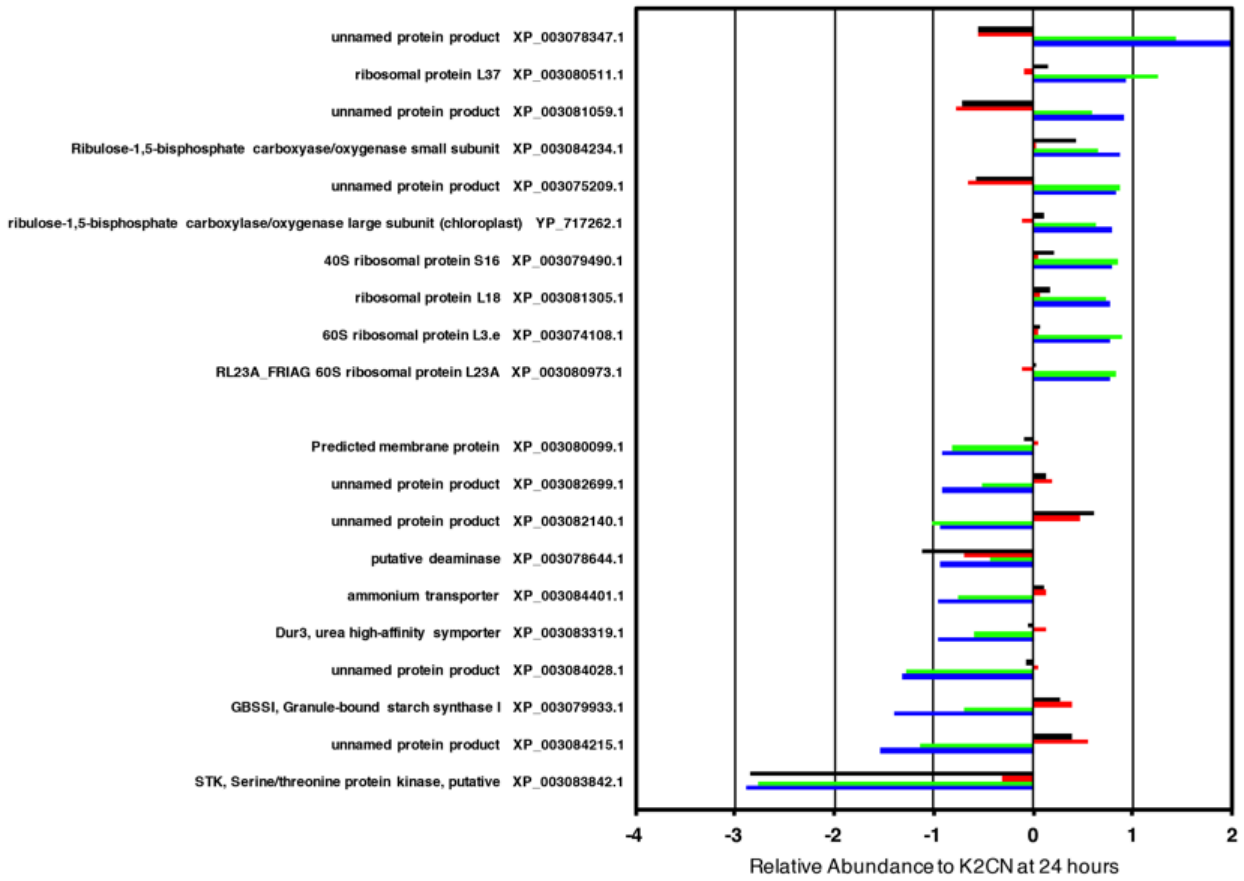


Figure 3 Selection differentially expressed proteins from global proteomics of varying N and C conditions
Abundance profiles for 10 most downregulated (positive values) and upregulated (negative values) proteins comparing K2CN at 24 hours versus K6CN at 48 hours (black), K2CN at 48 hours (red), K2C at 48 hours (green), and K6C at 48 hours (blue) cell cultures.

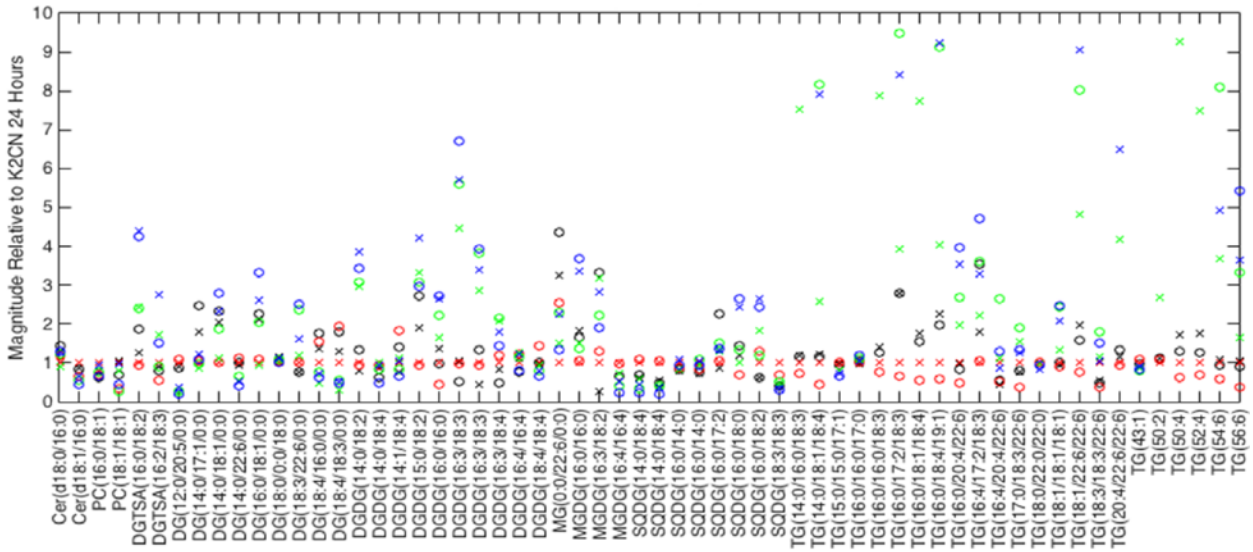
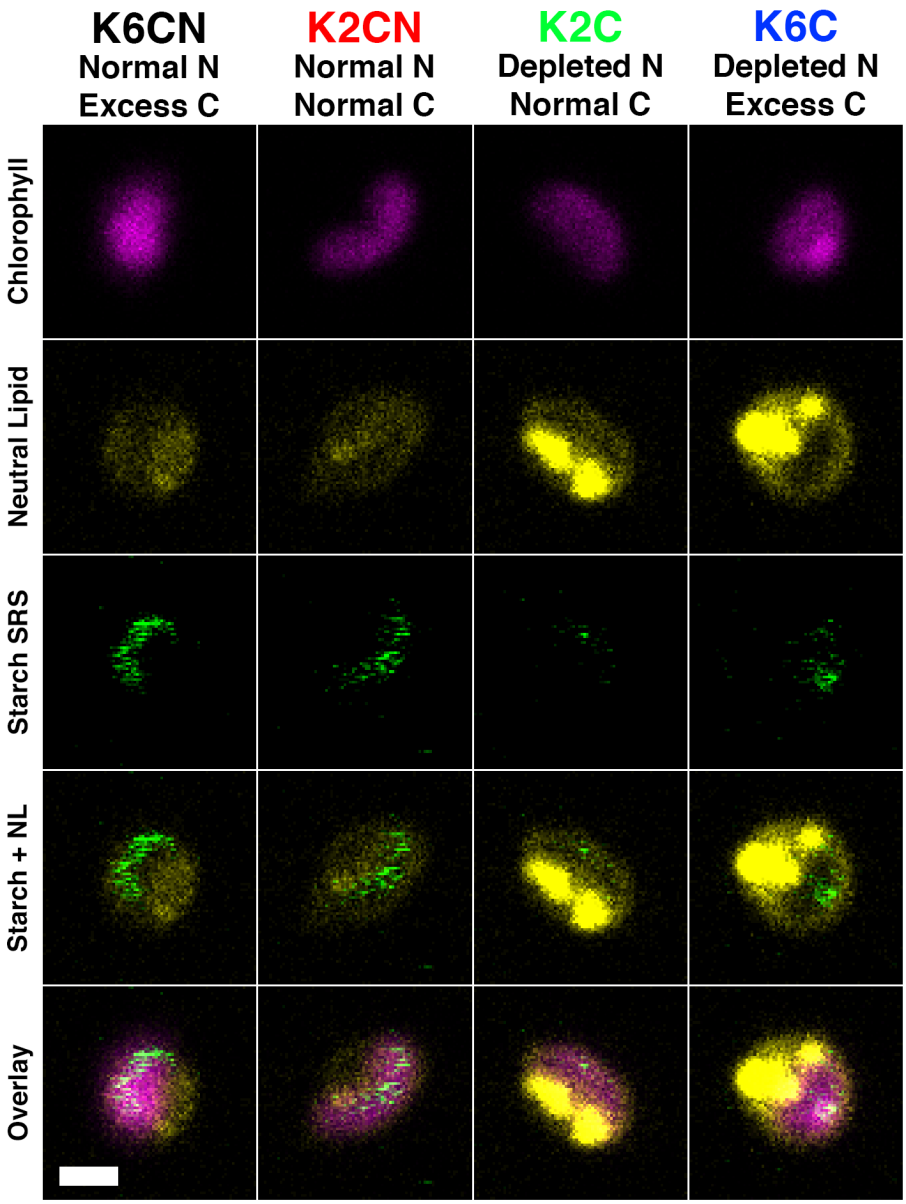


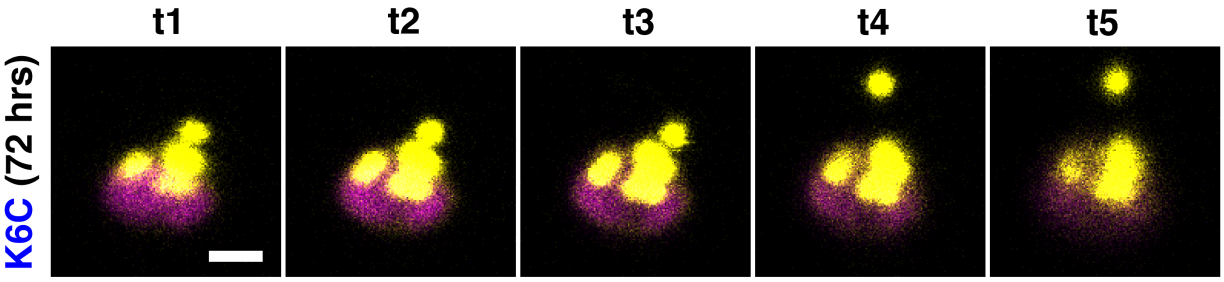
Figure 4 Time resolved differential expression of global lipids

Selection of lipids (out of 287) detected via LC-MS/MS for cell cultures at 24 (X symbols) and 48 hours (O symbols) for varying C:N ratio conditions K6CN (black), K2CN (red), K2C (green) and K6C (blue). *Cer: Ceramide; PC: phosphatidylcholine; MGDG: monogalactosyldiacylglycerol; DG: diacylglycerol; DGDG: digalactosyldiacylglycerol; DGTSA: diacylglyceryl trimethylhomoserine or diacylglyceryl trimethyl-beta-alanine; SQDG: sulfoquinovosyldiacylglycerol; and TG: triacylglycerols*

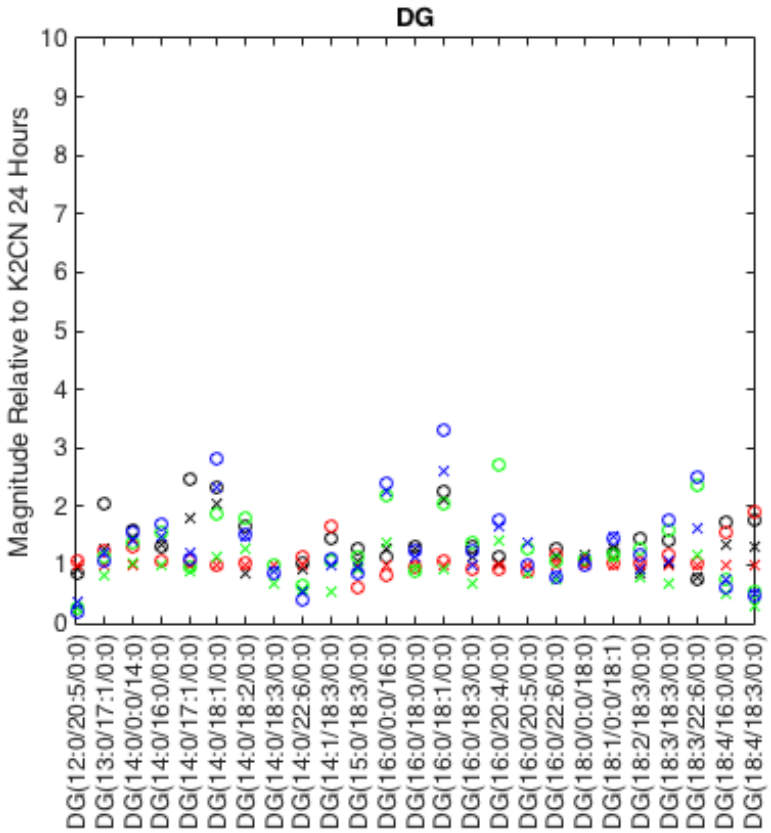
Supplemental Figures



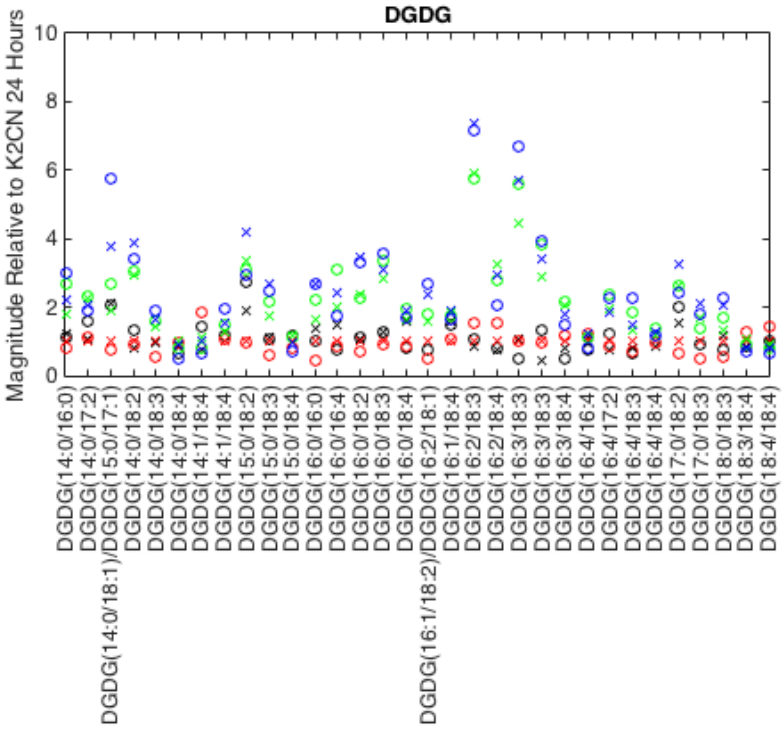
Supplemental Figure 1 Fluorescence and SRS verification of neutral lipid and starch accumulation
Fluorescence and SRS microscopy were conducted on *O. tauri* cells stained with Nile red from various C:N conditions after 96 hours of starvation. Chlorophyll autofluorescence (pink) was observed in all cells with neutral lipid (yellow) and SRS starch signal (green). Elevated neutral lipid was observed in K2C and K6C cells localized outside the chloroplast. Starch was detected and localized to the chloroplast for K6CN, K2CN, and to a lesser extent K6C, with little to no starch signal found in K2C cells. Scale bar represents 1µm.



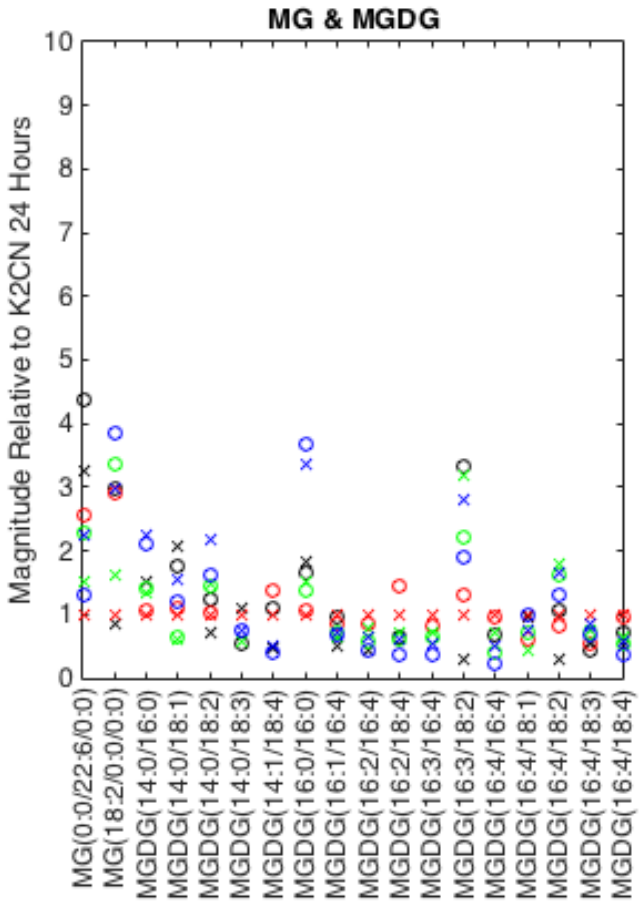
Supplemental Figure 2 Time course of lipid release from cells into intercellular space
O. tauri cells cultured in K6C media for 72 hours were harvested and stained with Nile red and then imaged by confocal fluorescence microscopy. Chlorophyll autofluorescence (pink) and neutral lipid (yellow) revealed many large lipid bodies during z-slice imaging and a single lipid body release was tracked over time (t) in intercellular milieu while other lipids remained inside the intracellular space.



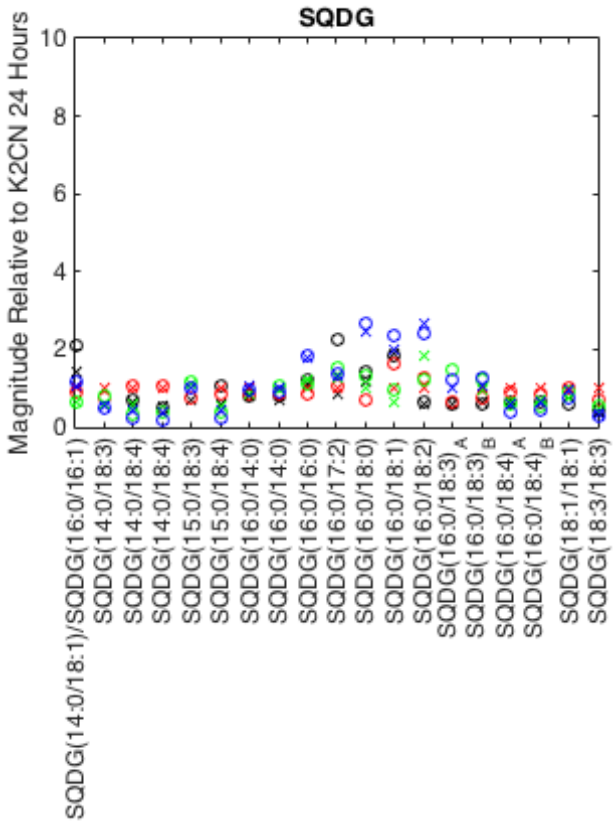
Supplemental Figure 3 Time-resolved lipidomics of diacylglycerol lipids
Diacylglycerol (DG) lipids detected via LC-MS/MS for cell cultures at 24 (X symbols) and 48 hours (O symbols) for varying C:N ratio conditions K6CN (black), K2CN (red), K2C (green) and K6C (blue) relative to K2CN at 24 hours (y-axis).



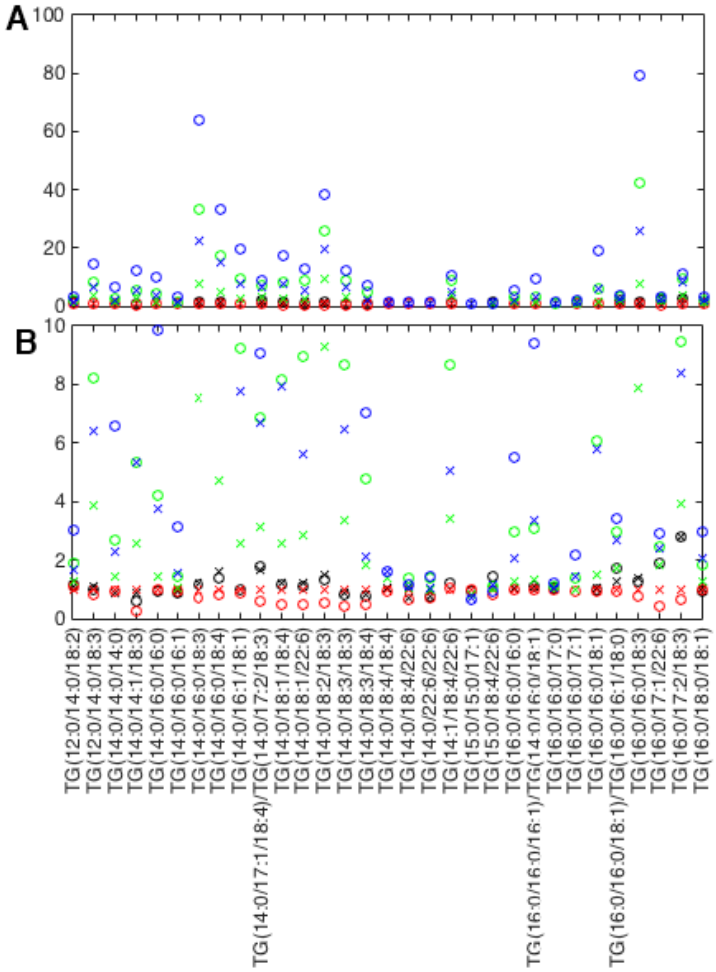
Supplemental Figure 4 Time resolved differential expression of digalactosyldiacylglycerol lipids
Digalactosyldiacylglycerol (DGDG) lipids detected for cell cultures at 24 (X symbols) and 48 hours (O symbols) for varying C:N ratio conditions K6CN (black), K2CN (red), K2C (green) and K6C (blue) relative to K2CN at 24 hours (y-axis).



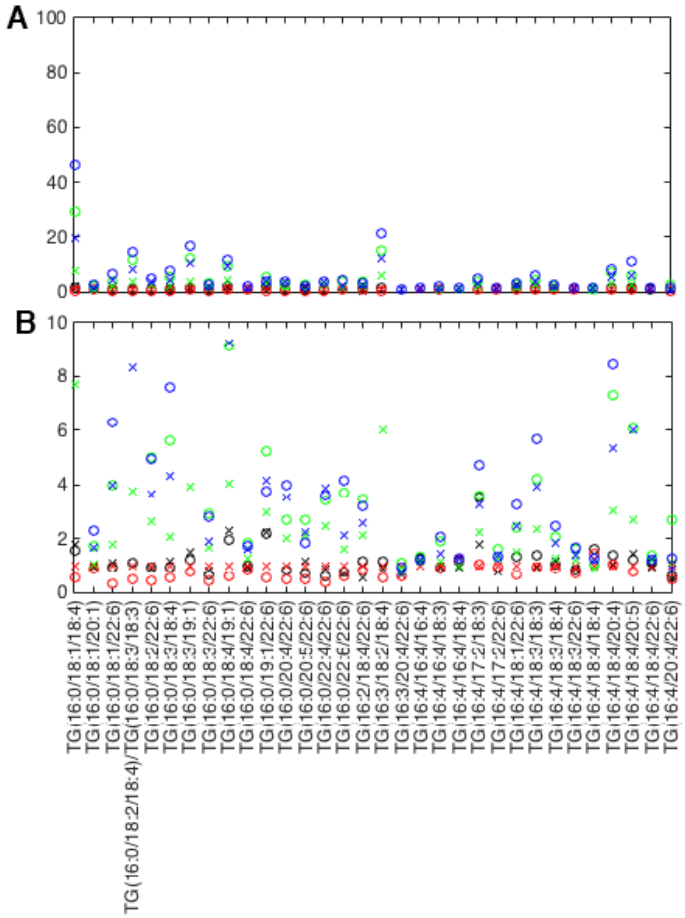
Supplemental Figure 5 Time resolved differential expression of monoacylglycerol and monogalactosyldiacylglycerol lipids
Monoacylglycerol (MG) and monogalactosyldiacylglycerol (MGDG) lipids detected via LC-MS/MS for cell cultures at 24 (X symbols) and 48 hours (O symbols) for varying C:N ratio conditions K6CN (black), K2CN (red), K2C (green) and K6C (blue) relative to K2CN at 24 hours (y-axis).



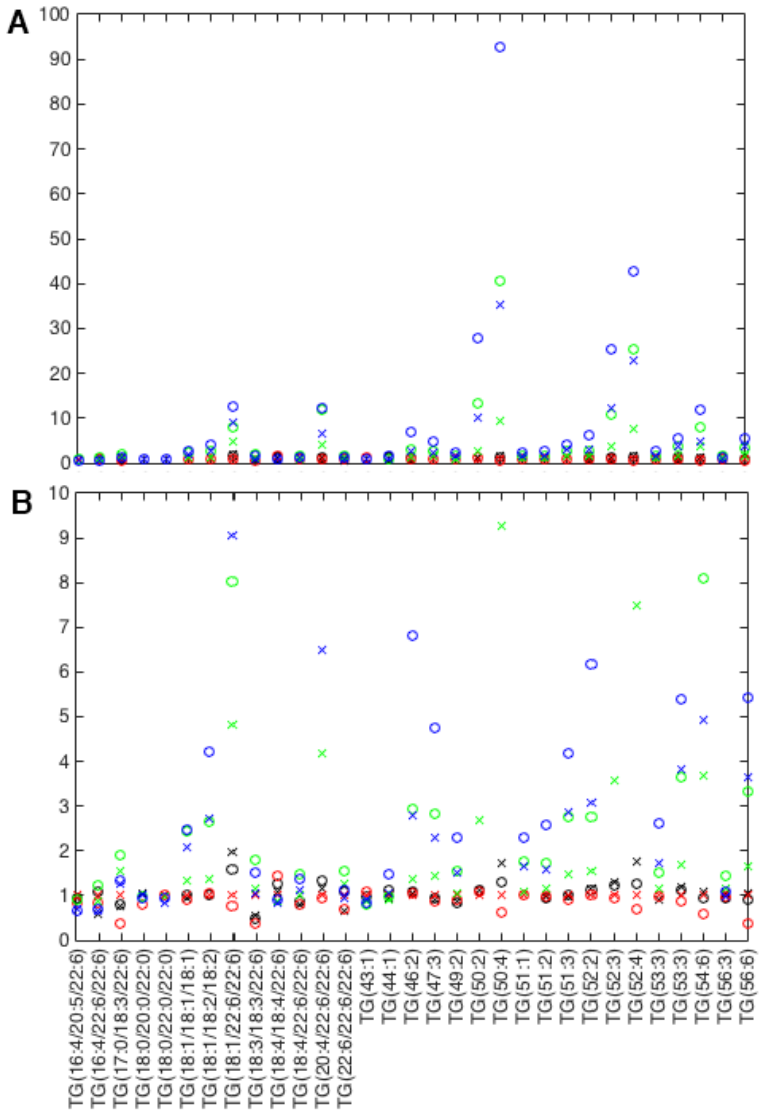
Supplemental Figure 6 Time resolved differential expression of lipids
Sulfoquinovosyldiacylglycerol (SQDG) lipids detected via LC-MS/MS for cell cultures at 24 (X symbols) and 48 hours (O symbols) for varying C:N ratio conditions K6CN (black), K2CN (red), K2C (green) and K6C (blue) relative to K2CN at 24 hours (y-axis).



Supplemental Figure 7 Time resolved differential abundance of triacylglycerol lipids set 1
Triacylglycerol (TG) lipidomics for cell cultures at 24 (X symbols) and 48 hours (O symbols) for varying C:N ratio conditions K6CN (black), K2CN (red), K2C (green) and K6C (blue) relative to K2CN at 24 hours (y-axis).



Supplemental Figure 8 Time resolved differential abundance of triacylglycerol lipids, set 2
Triacylglycerol (TG) lipidomics detected via LC-MS/MS for cell cultures at 24 (X symbols) and 48 hours (O symbols) for varying C:N ratio conditions K6CN (black), K2CN (red), K2C (green) and K6C (blue) relative to K2CN at 24 hours (y-axis).



Supplemental Figure 9 Time resolved differential abundance of long chain triacylglycerol lipids, scale 10
Long chain triacylglycerol (TG) lipidomics for cell cultures at 24 (X symbols) and 48 hours (O symbols) for varying C:N ratio conditions K6CN (black), K2CN (red), K2C (green) and K6C (blue) relative to K2CN at 24 hours (y-axis).