Electronic Supplementary Material (ESI) for Green Chemistry.
This journal is © The Royal Society of Chemistry 2019

1 # Electronic Supporting Information (ESI)

2

3 ## Towards sustainable microalgal biomass processing: Anaerobic
4 ## induction of autolytic cell-wall self-ingestion in lipid-rich
5 ## *Nannochloropsis* slurries

6

7 **Ronald Halim[a,b,c,+], David R.A. Hill[a], Eric Hanssen[d], Paul A. Webley[a], Susan Blackburn[e],**
8 **Arthur R. Grossman[b], Clemens Posten[c], Gregory J.O. Martin[a]**

9 [a] Algal Processing Group, Department of Chemical Engineering, The University of
10 Melbourne, Victoria, 3010, Australia
11 [b] Department of Plant Biology, Carnegie Institution for Science, Stanford University,
12 California, 94305, USA
13 [c] Institute of Process Engineering in Life Sciences, Bioprocess Engineering, Karlsruhe
14 Institute of Technology, Baden-Württemberg, 76131, Germany
15 [d] Advanced Microscopy Unit, Bio21 Molecular Science and Biotechnology Institute, The
16 University of Melbourne, Victoria, 3010, Australia
17 [e] The Australian National Algae Culture Collection, Commonwealth Scientific and Industrial
18 Research Organisation (CSIRO), Tasmania, 7004, Australia

19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34

35 **Supplementary Materials and Methods**

36

37 ***Microalgal cultivation:*** Two separate cultivation systems were used. With the first system
38 (exp. 1 – 16 in Table S1), monoculture of *Nannochloropsis* sp. strain was grown indoors in 15
39 L transparent blue carboys at room temperature under cool white illumination of 100-150
40 μmol/ m^2/ s with a 14:10 h light:dark cycle. The carboy was supplied with 0.21 vvm of
41 filtered compressed air. The system was operated semi-continuously with 80% of the working
42 volume harvested on day 14 and the remaining 20% replenished with fresh medium to an
43 OD_{750} of ~0.3 to restart cultivation. Under these conditions, nitrate was depleted by day 12.
44 Microalgal harvest was concentrated using a disc-stack centrifuge (Separator OTC 2-02-137,
45 GEA Westfalia, Germany).
46 With the second system (exp. 17 – 20 in Table S1), *N. gaditana* monoculture was grown
47 under axenic conditions in an indoor 25 L bubble-column photobioreactor (PBR) at 21ºC
48 with permanent white illumination (from 120 to 370 μmol / m^2/ s). The reactor was aerated
49 with 0.15 vvm of filtered CO_2-enriched air (CO2 : air = 1/100 v/v). To achieve axenic
50 cultivation, the PBR was filled with seawater and steam sterilised (100°C and 1 atm) for 20
51 min prior to the introduction of previously sterile-filtered medium components (0.22 μm) and
52 culture inoculum (prepared axenically). The PBR was harvested on day 14 (nitrate depleted
53 on day 10) and the harvest concentrated using a bucket-swing centrifuge.

54

55 ***Solid phase extraction (SPE):*** Aliquots (700 μl) of lipid solution (containing ~ 32 mg of
56 lipid) were separated into its constituent fractions (neutral lipid NL, glycolipid GL and
57 phospholipid PL) by sequential elution with different solvent systems in a SampliQ pre-
58 packed silica cartridge (Agilent Technologies, USA) as previously described [24].

59

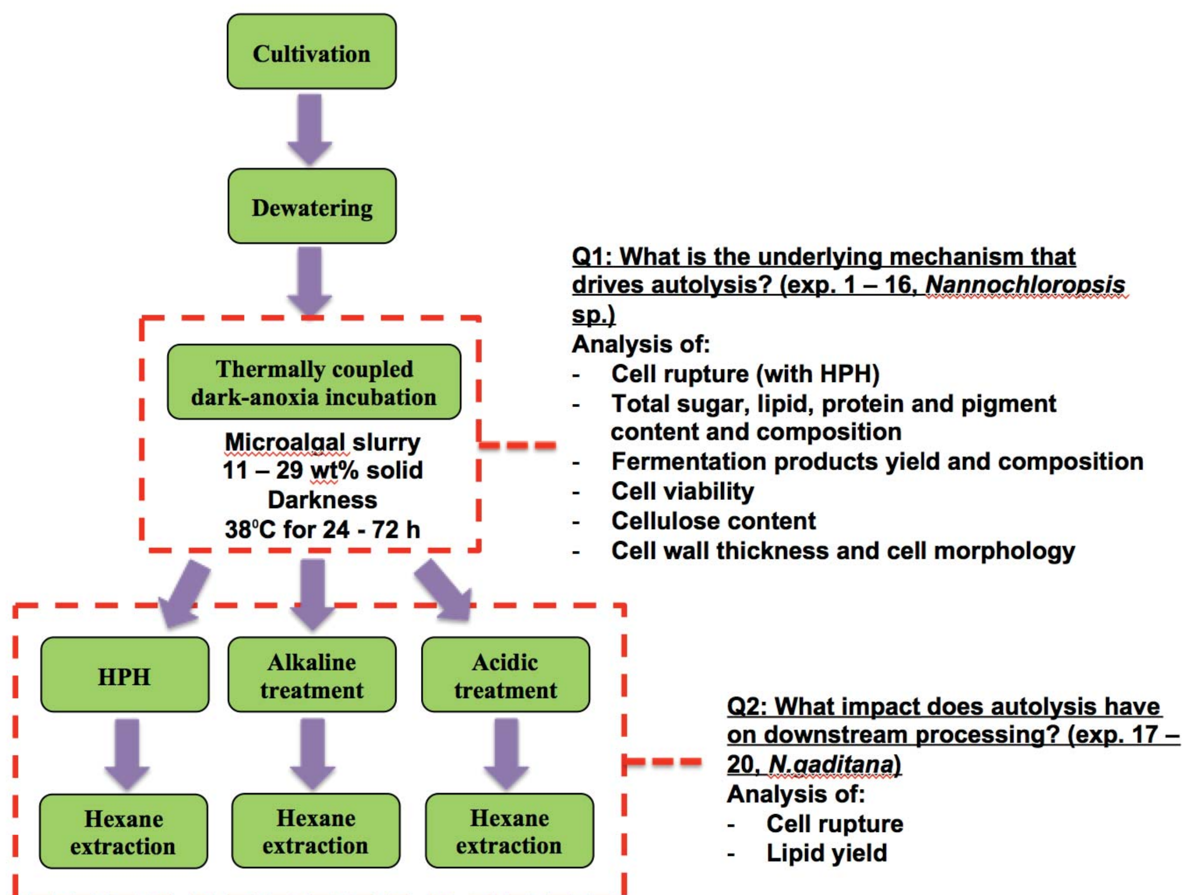
60 ***Lipid transesterification and gas chromatography:*** An aliquot (600 μl) of lipid solution
61 (containing ~27 mg of lipid) was mixed with 600 μl of methanol and 50 μl of C17:0 TAG
62 internal standard solution (containing 2.8 mg of TAG in chloroform). To this new solution,
63 300 μl of 10 wt% H_2SO_4 in methanol was then added. The acid methylation reaction, intended
64 to esterify free fatty acids (FFA) and to transesterify triacylglycerols (TAG), was carried out
65 at 55°C and 200 rpm for 3 h. At the end of the acid methylation step, 1 ml of 25 wt% KCH_3O
66 in methanol was added to initiate the alkaline methylation step (pH value after alkaline
67 addition = 13). Alkaline methylation, carried out at 55°C and 200 rpm for 2 h, was intended
68 to quench any interfering water contaminants and to completely esterify TAG and polar
69 lipids. For both methylation steps, the reaction vial was sealed to prevent solvent evaporation.
70 At the end of alkaline methylation, 40 μl of C15:0 FAME internal standard solution
71 (containing 1.3 mg of FAME in chloroform) was added to the solution. FAME solution was

72 filtered (0.2 μm nylon syringe filter) to remove any solid precipitate formed during the
73 methylation reactions and stored at -20°C.
74 4 μl of the FAME solution was manually injected into an Agilent 7890B GC-FID unit
75 (Agilent Technologies, USA) equipped with a DB23 methylpolysiloxane capillary column
76 (30 m x 0.32 mm id x 0.25 μm) (Agilent Technologies, USA). Helium was used as the carrier
77 gas at a split ratio of 80:1 and a flow rate of 56.171 ml/min . The detector temperature was set
78 at 300°C. The following oven configuration was used: an initial temperature at 50°C for 1
79 min, a linear ramp at 20°C/min to 180°C and a second linear ramp at 2°C/min to 210°C.
80 Individual FAME was identified by retention-time comparison with a mixed FAME 18917
81 Supelco standard (Sigma Aldrich, USA) and quantified using a 4-point linear calibration
82 curve.
83
84 ***Amino acid analysis:*** Lyophilised and ground biomass powder (50 – 100 mg) of untreated or
85 incubated cells were added to 2.5 mL of 20% HCl, flushed with nitrogen and heated at 110°C
86 for 24h. The hydrolysate was spiked with norvaline (internal standard) and diluted 25x prior
87 to 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatisation with AccQ-Tag
88 Ultra derivatisation kit (Waters Corporation, USA) in accordance to the supplier's manual.
89 Chromatographic separation and quantitation of 18 free amino acids in the hydrolysate were
90 performed on an ACQUITY Ultra- Performance Liquid Chromatography unit (Waters
91 Corporation, USA) with a UV detection system (260 nm) and a BEH RP C18 column (2.1 x
92 100 mm, 1.7 μm) (Waters Corporation, USA) at 57°C.
93
94 ***Total pigment analysis:*** Microalgal slurry was weighed in a small glass mortar before being
95 ground with 2-3 ml of 100% acetone. The mixture was sonicated in an ice-water bath for 20
96 min and left at 4 °C for approximately 15 h. Both sonication and resting period took place in
97 darkness. The mixture was pelleted via centrifugation and the supernatant decanted. The
98 extraction cycle was repeated with a shorter resting period of 3 h. To the pooled supernatant
99 from both steps, water was added to make a solution with a final composition of 90:10
100 acetone:water (v/v). Pigment solution was filtered (0.2 μm) prior to analysis using a Waters-
101 Alliance HPLC system that comprised of a 2695XE separations module, a Zorbax Eclipse
102 XDB-C8 stainless-steel 150 mm x 4.6 mm ID column with 3.5 μm particle size (Agilent
103 Technologies, USA), a column heater and a 2996 photo-diode array detector. The mobile
104 eluent was 28 mM tetrabutyl ammonium acetate: methanol mixture.
105
106 ***SEM:*** Coverslips (22 mm glass) were smeared with 0.1% (v/v) polyethyleneimine (PEI) and
107 dried under a flame. Cells (200 μL) were placed on the PEI-coated glass coverslips for 1 h.
108 The excess culture was drained and coverslips with adhered cells were further immersed in

2.5% glutaraldehyde in PBS for 1 h. After immersion, the coverslips were dehydrated in increasing concentrations of ethanol consisting of 10, 30, 50, 70, 90 and 100% ethanol in water for 10 min each step. The coverslips were then dried in a Balzers CPD030 critical point dryer (Balzers, Germany) before being mounted onto 25 mm aluminium stubs with double-sided carbon tabs and coated with gold using a Xenosput sputter coater (Dynavac, Australia). A Philips XL30 field-emission scanning electron microscope (Philips, the Netherlands) at a voltage of 2.0 kV and spot size of 2 was used to image the cells.

146

Supplementary Figures and Tables



147 Figure S1. Experimental scheme for the study. The study has two distinct objectives. For the
148 first objective, we studied the underlying fermentation mechanism that triggered the induction
149 of autolysis in *Nannochloropsis* cells. For the second objective, we investigated the impact of
150 autolysis on downstream processing (extent of cell rupture and lipid extraction yield). Several
151 pathways (HPH, alkali treatment and acid treatment) were examined.

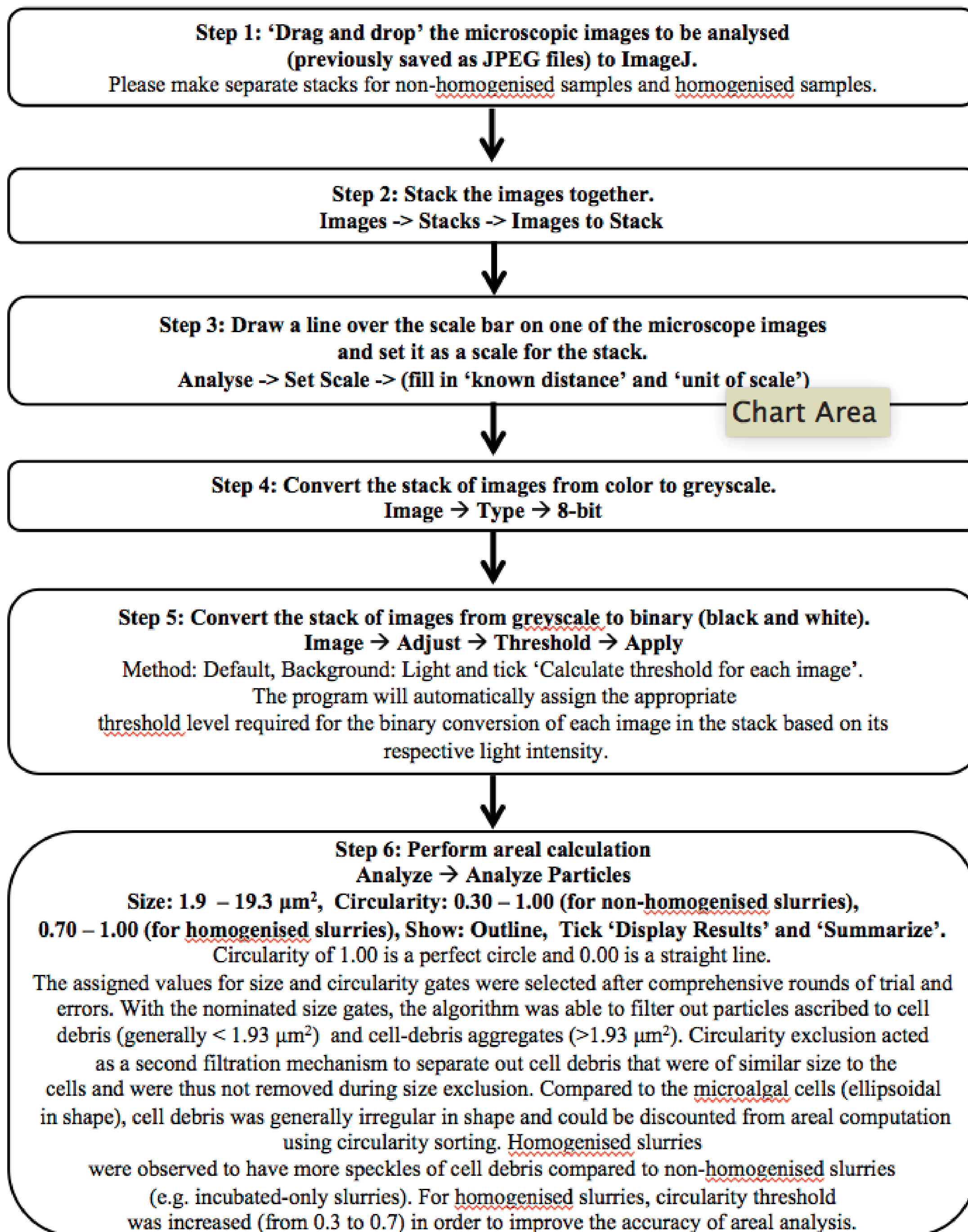
152

153

154

155

156



157

158 Figure S2. Algorithm for automated image analysis with ImageJ Software. The algorithm was
159 able to selectively calculate the area occupied by whole cells on the microscope image
160 (*apparent cell area*) while filtering out the area attributed to cell debris and agglomerates.

161

162

163

164

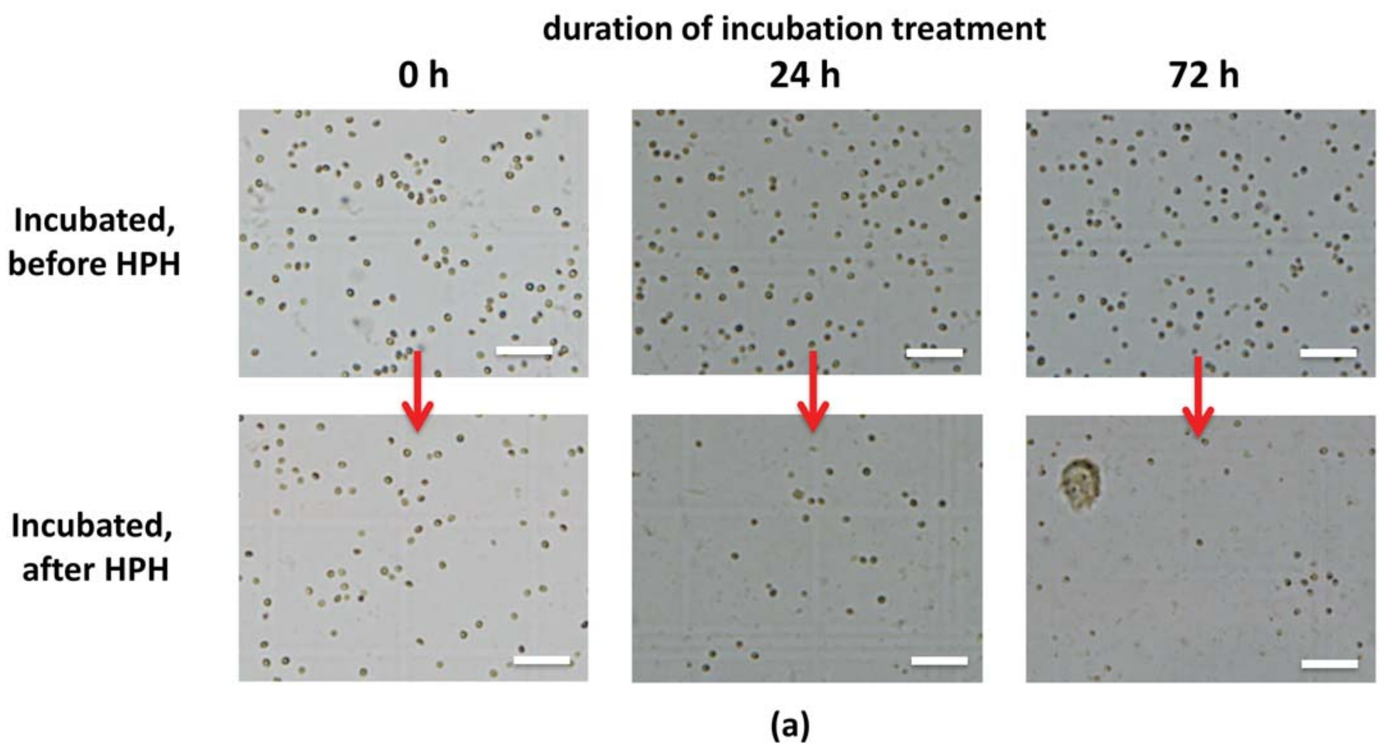
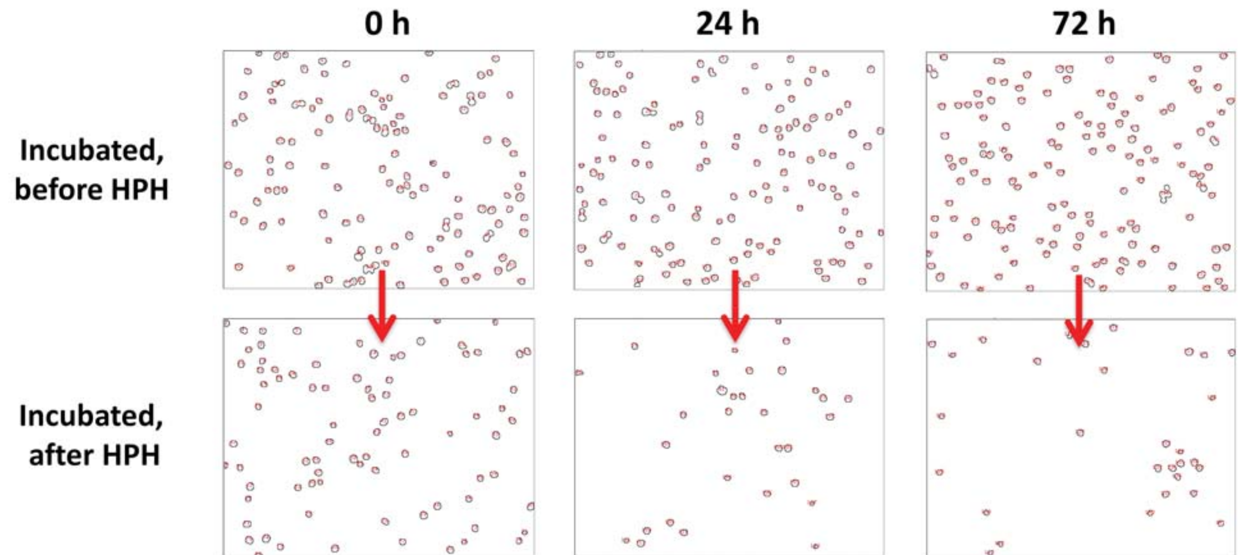


Figure S3. (a). Microscopic images of *Nannochloropsis* sp. cells. The ability of thermally coupled dark-anoxia incubation in inducing autolysis in *Nannochloropsis* cells and thus enhancing the degree of cell rupture after high-pressure homogenisation was illustrated. Since cells in the untreated slurry (0 h) were more resistant to cell rupture, more of them remained intact after being subjected to HPH. On the other hand, cells in the post-incubated cells (24 h and 72 h) were more vulnerable to cell rupture; less intact cells were seen under the microscope after HPH. The slides were extracted from the results of experiment 1 (incubation at 38°C and 120 rpm).

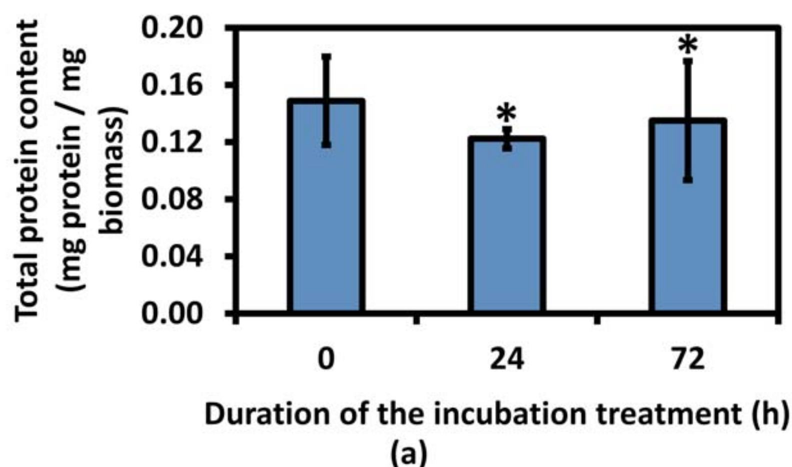


Analysis of microscopic images	The duration of incubation treatment					
	0 h		24 h		72 h	
	Before homogenisation	After homogenisation	Before homogenisation	After homogenisation	Before homogenisation	After homogenisation
No. of counts	117	82	128	31	138	30
Apparent cell area (μm^2)	711	432	696	154	722	133
Cell rupture (% of available cells)		39.2		78.3		81.3

$$\text{Extent of cell rupture (\% of available cells)} = \frac{\text{Apparent cell area before HPH } (\mu m^2) - \text{Apparent cell area after HPH } (\mu m^2)}{\text{Apparent cell area before HPH } (\mu m^2)}$$

(b)

Figure S3. (b). Cell rupture evaluation of the microscopic images shown in Figure S3(a) using automated imageJ analysis algorithm described Figure S2. The ImageJ analysis generated binary version of the microscopic images, while calculating the number of particle counts and the total amount of area attributed only to whole microalgal cells (apparent cell area). Scale bar (a) = 20 μm.



(a)

Amino acid	untreated cells (wt% of total amino acid)	24-h incubated cells (wt% of total amino acid)	72-h incubated cells (wt% of total amino acid)
Histidine	2.29 ± 0.02	2.27 ± 0.05	2.27 ± 0.03
Serine	5.05 ± 0.09	4.99 ± 0.08	5.01 ± 0.06
Arginine	6.06 ± 0.28	6.17 ± 0.13	6.22 ± 0.15
Glycine	6.66 ± 0.17	6.80 ± 0.14	6.86 ± 0.14
Asparagine and Aspartic acid	9.93 ± 0.13	9.98 ± 0.09	9.84 ± 0.08
Glutamine and Glutamic acid	12.03 ± 0.15	11.70 ± 0.04	11.42 ± 0.15
Threonine	5.85 ± 0.26	5.92 ± 0.23	5.99 ± 0.23
Alanine	7.51 ± 0.12	7.34 ± 0.08	7.42 ± 0.09
Proline	5.11 ± 0.01	5.19 ± 0.01	5.26 ± 0.00
Lysine	7.12 ± 0.14	6.78 ± 0.36	6.74 ± 0.31
Tyrosine	3.03 ± 0.25	3.22 ± 0.19	3.28 ± 0.19
Methionine	2.49 ± 0.04	2.52 ± 0.03	2.53 ± 0.04
Valine	6.88 ± 0.08	6.93 ± 0.10	6.96 ± 0.09
Isoleucine	4.94 ± 0.01	4.96 ± 0.05	5.01 ± 0.02
Leucine	9.61 ± 0.02	9.65 ± 0.04	9.64 ± 0.01
Phenylalanine	5.47 ± 0.07	5.59 ± 0.17	5.56 ± 0.14
Total	100.00	100.00	100.00

(b)

205

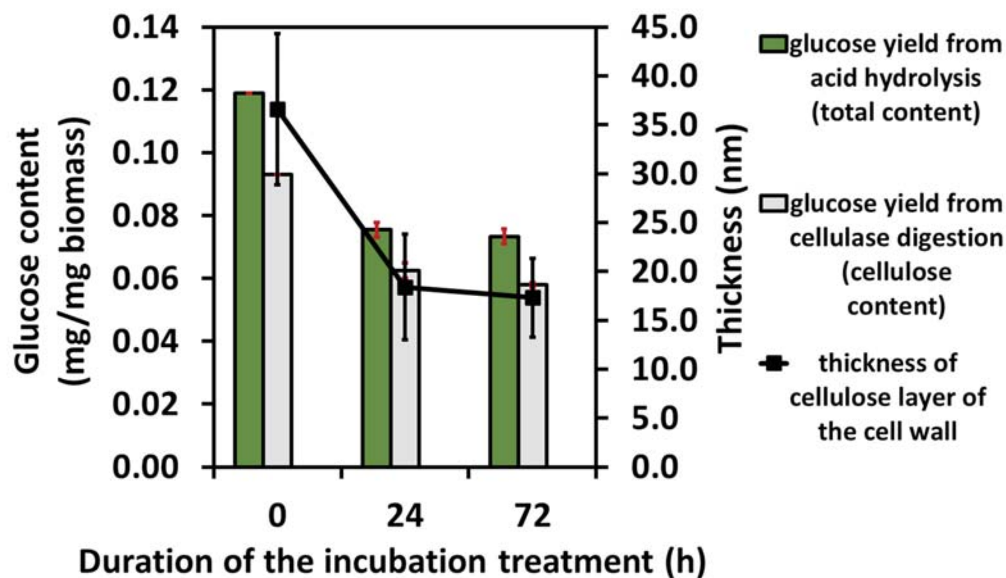
206 Figure S4. (a) Total protein content of *Nannochloropsis* sp. biomass as a function of
207 incubation time. In order to evaluate total protein, the biomass was subjected to NaOH
208 hydrolysis followed by Lowry's analysis of the hydrolysate. (b) Amino acid composition of
209 *Nannochloropsis* sp. biomass as a function of incubation time. In order to evaluate amino acid
210 composition, the biomass was subjected to HCl hydrolysis followed by HPLC analysis of the
211 hydrolysed and derivatised free amino acids. Both Figures present results (mean ± std) for
212 incubation at 38°C and 0 rpm: exp. 14, 15 and 16. In Figure a, * denotes statistical
213 significance (p-value < 0.01). For Figure b, Cysteine and Tryptophan were destroyed by the
214 acid treatment and thus not included in the composition analysis.

215

216

217

218



219

220 Figure S5. The total glucose content and total cellulose content of *Nannochloropsis* sp.
221 biomass as well as the thickness of the cellulose layer of the cell wall as a function of
222 incubation time. Values for total glucose content were extracted from the results of total sugar
223 analysis. In order to evaluate cellulosic glucose content (glucose yield attributed only to
224 cellulose), the biomass was subjected to enzymatic digestion (with cellulase) followed by
225 HPLC (at the RI detector) analysis of the released glucose in the hydrolysate. The Figure
226 presents results (mean ± std) for incubation at 38°C and 60 - 120 rpm: exp. 11, 12, 13.

227

228

229

230

231

232

233

234

235

236

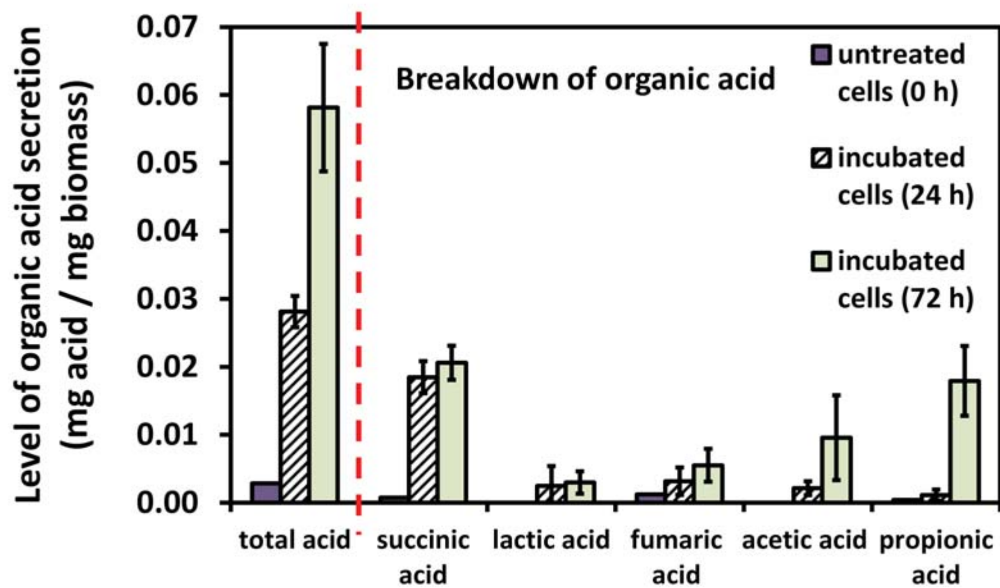
237

238

239

240

241

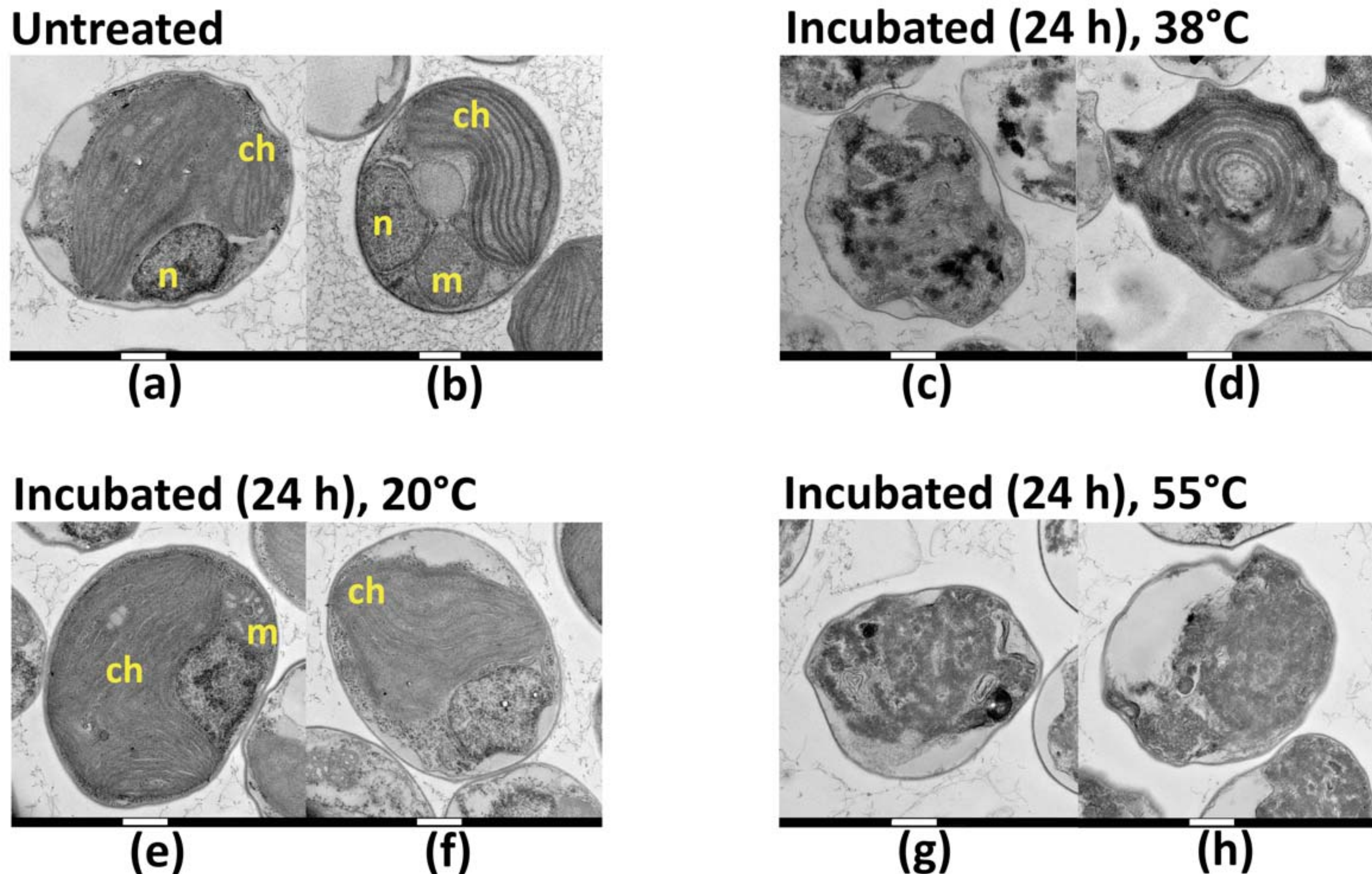


242

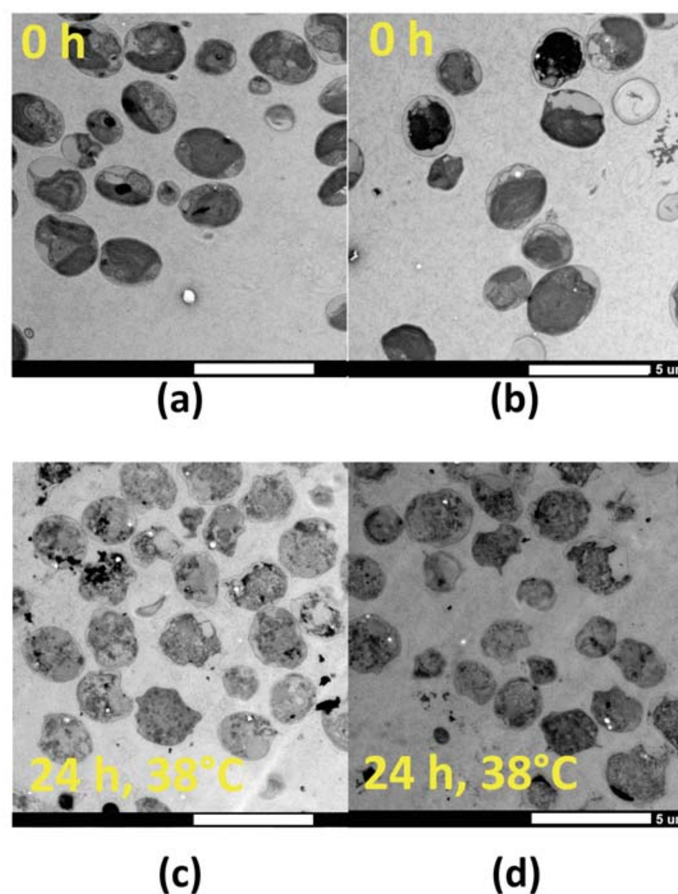
243 Figure S6. The amount and profile of organic acids secreted by *Nannochloropsis* sp. cells
244 autolysis. In order to obtain organic acid composition, we washed microalgal slurry collected
245 at different time points of the incubation treatment with DI water and analysed the wash
246 solution with HPLC at the UV-Vis detector. Only 5 of the most abundant organic acids are
247 shown. The Figure presents results (mean ± std) for incubation at 38°C and 60 - 120 rpm:
248 exp. 1, 2, 3.

249

250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267



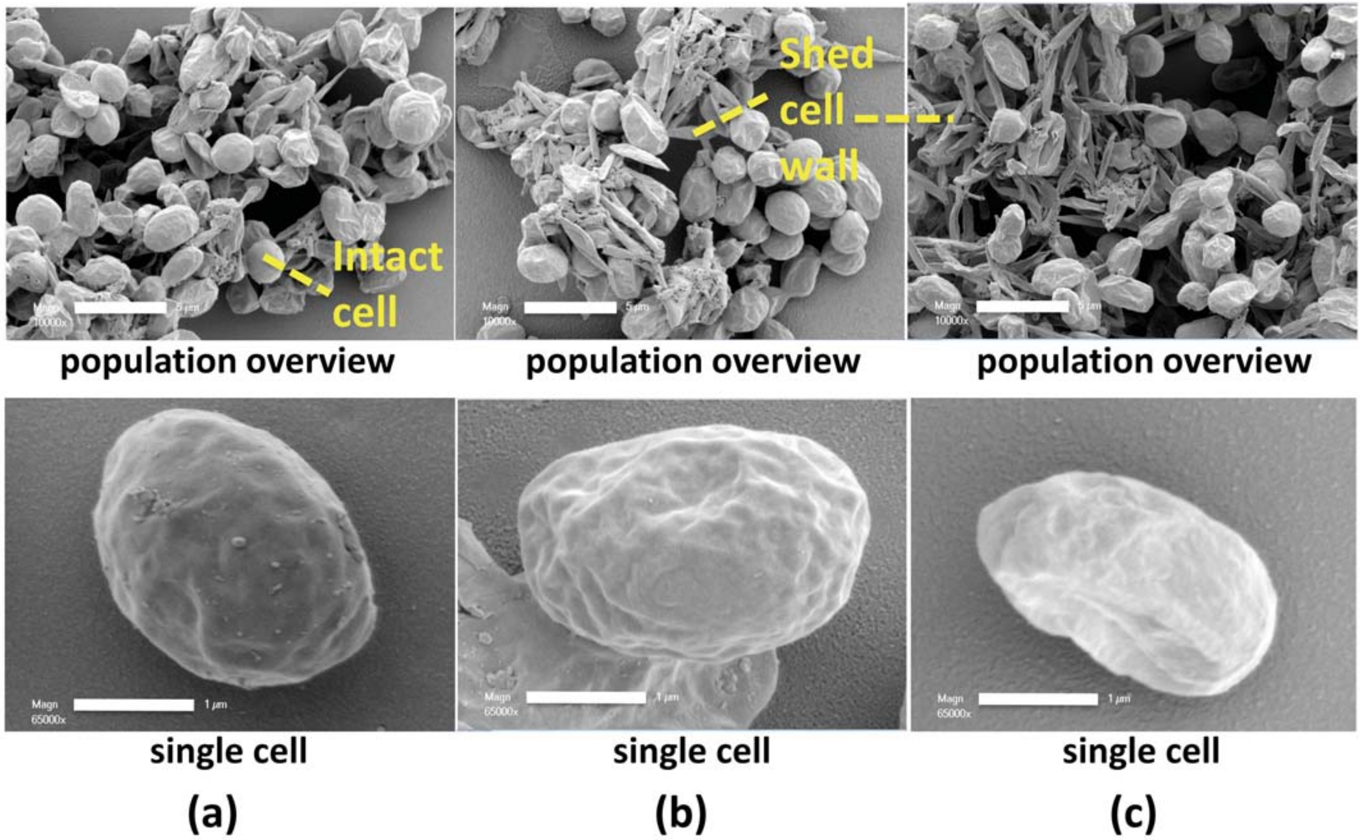
268 Figure S7. . Representative TEM images of whole *Nannochloropsis* sp. cells (untreated and incubated). (a) untreated (exp. 1). (b) untreated (exp. 12). (c) 24-
269 h incubated at 38°C and 120 rpm (exp. 1). (d) 24-h incubated at 38°C and120 rpm (exp. 1). (e) 24-h incubated at 20°C and 120 rpm (exp. 6). (f) 24-h
270 incubated at 20°C and 120 rpm (exp. 6). (g) 24-h incubated at 55°C and 120 rpm (exp. 8). (h) 24-h incubated at 55°C and 120 rpm (exp. 8).
271 Scale bar (a – l) = 500 nm. n: nucleus, m: mitochondrion, ch: chloroplast



Experiment no.	Temperature (°C)	Agitation (rpm)	Circularity of cells 0 h	Circularity of cells 24 h
1	38	120	0.56 ± 0.17	0.28 ± 0.10

(e)

Figure S8. Representative TEM images showing overviews of the population of untreated and incubated *Nannochloropsis* sp. cells. (a) untreated, exp. 1. (b) untreated, exp. 1. (c) 24-h incubated (38°C, 120 rpm), exp. 1 (d) 24-h incubated (38°C, 120 rpm), exp. 1. Scale bar (a-d) = 5 µm. (e) Summary table outlining the shape (circularity) of *Nannochloropsis* sp. cells as a function of incubation time. TEM images were analysed with ImageJ software in order to generate the shape analysis.

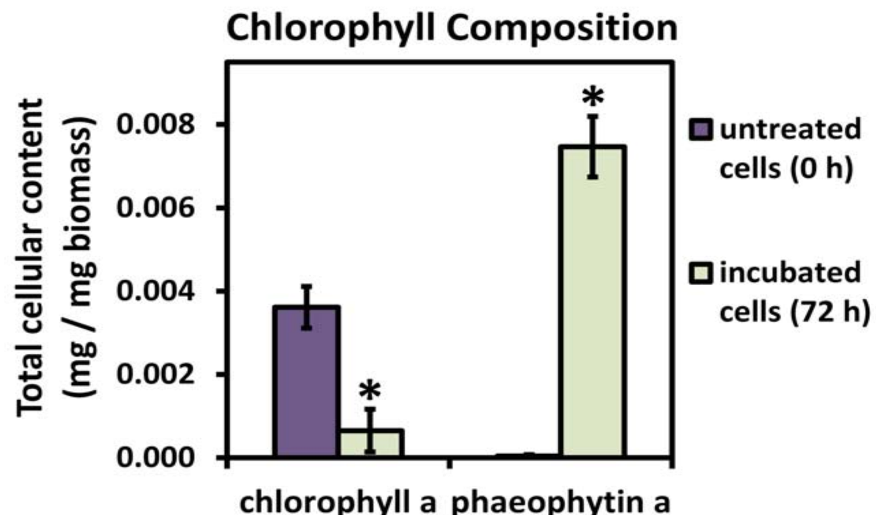


285

286 Figure S9. SEM images of untreated and incubated *Nannochloropsis* sp. cells. (a) untreated,
287 exp. 13. (b) 24-h incubated (38°C, 120 rpm), exp. 13. (c) 72-h incubated (38°C, 120 rpm),
288 exp. 13. Scale bar (a-c population) = 5 µm. Scale bar (a-c single cell) = 1 µm.

289
290
291
292
293
294
295
296
297
298
299
300
301
302
303

(a)



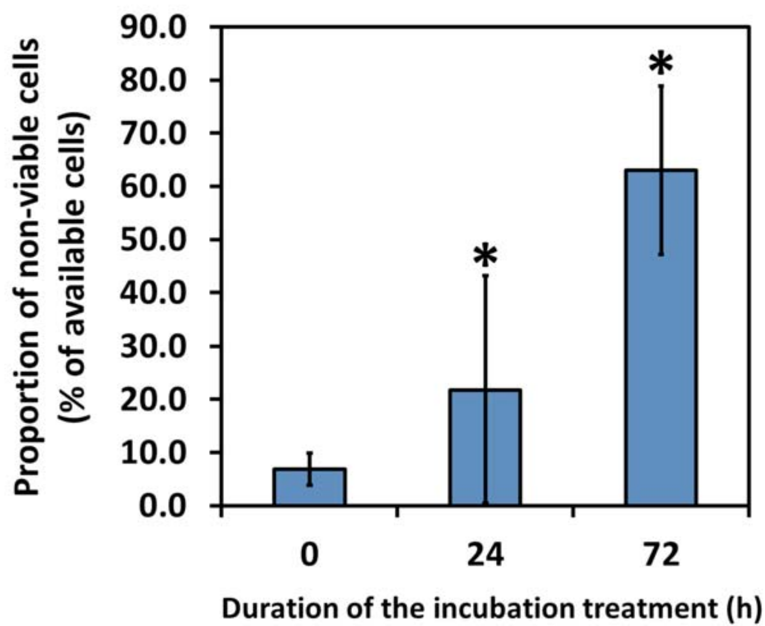
(b)

Pigment	Total cellular content (mg / mg biomass)		Net change
	Untreated cells	Incubated cells (72 h)	
antheraxathin	0.00001 ± 0.00001	0.00000 ± 0.00000	
chlorophyll a	0.00361 ± 0.00050	0.00065 ± 0.00051	
chlorophyllide a	0.00004 ± 0.00002	0.00000 ± 0.00000	-
vauchelarxanthin	0.00025 ± 0.00003	0.00006 ± 0.00005	
violaxanthin	0.00054 ± 0.00006	0.00000 ± 0.00000	
echnininone	0.00001 ± 0.00000	0.00003 ± 0.00001	
phaeophytin a	0.00004 ± 0.00003	0.00747 ± 0.00073	+
vauchelarxanthin breakdown product	0.00001 ± 0.00001	0.00012 ± 0.00003	
zeaxanthin	0.00003 ± 0.00001	0.00011 ± 0.00008	
bb-carotene	0.00014 ± 0.00003	0.00014 ± 0.00008	=

(c)

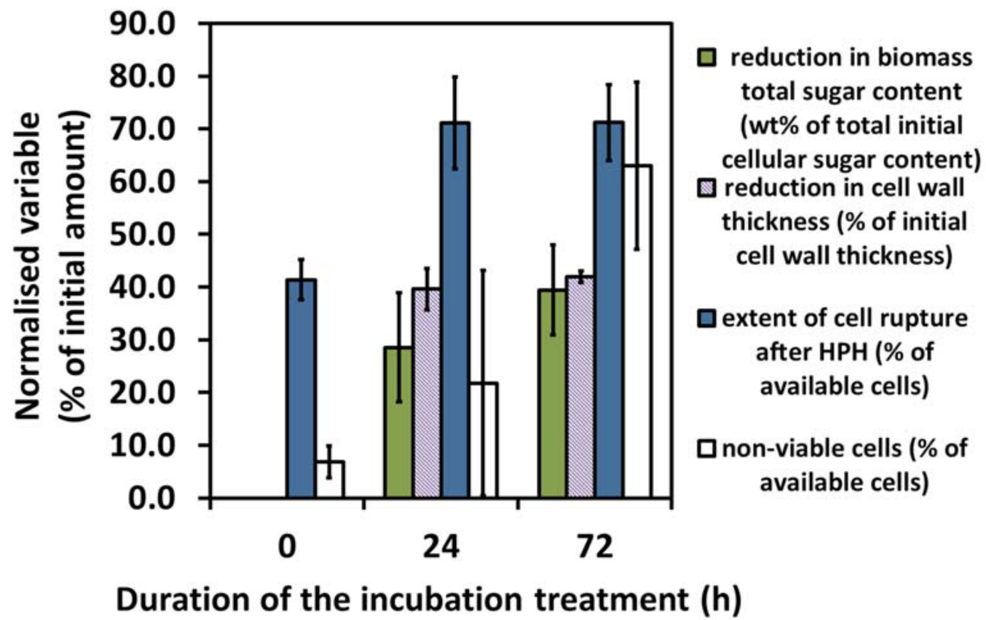
324 Figure S10. (a) The appearance of *Nannochloropsis* sp. slurry before and after 24 h of incubation (38°C, 120 rpm). (b) The effect of thermally coupled dark-
325 anoxia incubation on the chlorophyll *a* content of *Nannochloropsis* sp. biomass. (b) The effect of thermally coupled dark-anoxia incubation on the total
326 pigment content and composition of *Nannochloropsis* sp. biomass. In order to evaluate total pigment content and composition, the biomass was subjected to
327 ultrasound-assisted acetone extraction followed by HPLC analysis of the extracted pigment. Figure b & c present results (mean ± std) for incubation at 38°
328 and 120 rpm: exp. 1, 10, 12. In Figure b, * denotes statistical significance (p-value < 0.01).

329
330
331
332
333
334
335
336
337
338
339
340
341
342



343 Figure S11. The viability of *Nannochloropsis* sp. cells as a function of incubation time. Cell
344 viability was assessed with Sytox green staining followed by flow-cytometer analysis. The
345 Figure presents results (mean ± std) for incubation at 38°C and 0 rpm: exp. 1, 2, 3, 4, 5, 9, 10,
346 11, 12, 13. * denotes statistical significance (p-value < 0.01).

347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364



365

366 Figure S12. Comparison of the kinetics of critical parameters associated with autolysis. The
367 Figure presents results (mean ± std) for incubation at 38°C and 0 rpm: exp. exp. 1, 2, 3, 4, 5,
368 9, 10, 11, 12, 13.
369
370
371
372
373
374
375
376
377
378

379 Table S1. Summary of the experimental design used in the study.

380 (a) Summary of the microalgal strain and the parameters used for thermally coupled dark-anoxia incubation. (b) Outline of the analyses used to investigate the
381 mechanism of autolysis. (c) Outline of the analyses used to study the impact of autolysis on downstream processing.

382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402

403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420

	Experiment no.	Strain	Incubation treatment parameters		
			Temperature (°C)	Agitation (rpm)	Biomass concentration (g biomass / g slurry)
Experiments investigating the mechanism of autolysis	1	*Nannochloropsis* sp.	38	120	0.23
	2	*Nannochloropsis* sp.	38	120	0.23
	3	*Nannochloropsis* sp.	38	0	0.23
	4	*Nannochloropsis* sp.	38	60	0.29
	5	*Nannochloropsis* sp.	38	60	0.16
	6	*Nannochloropsis* sp.	20	120	0.23
	7	*Nannochloropsis* sp.	20	0	0.23
	8	*Nannochloropsis* sp.	55	120	0.23
	9	*Nannochloropsis* sp.	38	60	0.26
	10	*Nannochloropsis* sp.	38	120	0.26
	11	*Nannochloropsis* sp.	38	60	0.26
	12	*Nannochloropsis* sp.	38	120	0.26
	13	*Nannochloropsis* sp.	38	120	0.26
	14	*Nannochloropsis* sp.	38	0	0.29
	15	*Nannochloropsis* sp.	38	0	0.29
	16	*Nannochloropsis* sp.	38	0	0.29
Experiments investigating the impact of autolysis on downstream processing pathways	17	*N. gaditana* (SAG 2.99)	38	0	0.18
	18	*N. gaditana* (SAG 2.99)	38	0	0.15
	19	*N. gaditana* (SAG 2.99)	38	0	0.13
	20	*N. gaditana* (SAG 2.99)	38	0	0.15

(a)

Experiment no.	Mechanism of autolysis										
	Cell rupture with HPH	Total sugar analysis	Total lipid and fatty acid analysis	Total protein analysis (elemental analysis)	Total protein (Lowry's method) and amino acid analyses	Fermentation products analysis	Evaluation of cell wall thickness & cell morphology (TEM)	Evaluation of cell morphology (SEM)	Total pigment analysis	Cellulose analysis	Cell viability measurement
1	☑	☑	☑	☑		☑	☑		☑		☑
2	☑	☑		☑		☑					☑
3	☑	☑		☑		☑	☑				☑
4	☑	☑				☑					☑
5	☑	☑				☑					☑
6	☑	☑		☑		☑	☑				☑
7	☑					☑					☑
8	☑	☑		☑		☑	☑				☑
9	☑	☑		☑		☑					☑
10	☑	☑	☑	☑		☑	☑		☑		☑
11	☑	☑		☑		☑				☑	☑
12	☑	☑	☑	☑		☑	☑		☑	☑	☑
13	☑	☑		☑		☑		☑		☑	☑
14					☑						
15					☑						
16					☑						
17											
18											
19											
20											

421

(b)

422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440

Experiment no.	Impact of autolysis on downstream processing pathways					
	Cell rupture with HPH	Lipid extraction of HPH pathway (hexane)	Cell rupture with alkali	Lipid extraction of alkaline pathway (hexane)	Cell rupture with acid	Lipid extraction of acidic pathway (hexane)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17	☑					
18	☑	☑	☑	☑		
19	☑	☑	☑	☑		
20	☑	☑	☑	☑	☑	☑

441 (c)

442 Table S2. Complete set of results for all experiments in the study.

443 (a) Results for the investigation into autolysis mechanism: cell rupture with HPH, total sugar analysis and total protein (elemental) analysis. (b) Results for 444 the investigation into autolysis mechanism: total protein analysis (Lowry), total lipid and fatty acid analysis. (c) Results for the investigation into autolysis 445 mechanism: fermentation product analysis. (d) Results for the investigation into autolysis mechanism: measurement of cell wall thickness, cellulose analysis 446 and cell viability analysis. (e) Results for the investigation into the impact of autolysis on different downstream processing pathways: cell rupture with HPH, 447 alkali or acid, lipid extraction with HPH, alkali or acid.

Experiment no.	Cell rupture analysis			Total sugar analysis (HPLC)												Total protein analysis (elemental analysis)		
	Extent of cell rupture after HPH (% of available cells)			Total sugar content (mg / mg biomass)			Glucose content (mg / mg biomass)			Galactose content (mg / mg biomass)			Rhamnose content (mg / mg biomass)			Total protein content (mg / mg biomass)		
	duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)		
	0	24	72	0	24	72	0	24	72	0	24	72	0	24	72	0	24	72
1	36.8 ± 5.8	79.4 ± 1.1	73.1 ± 3.1	0.161 ± 0.005	0.112 ± 0.004	0.096 ± 0.002	0.091 ± 0.003	0.070 ± 0.002	0.063 ± 0.001	0.028 ± 0.002	0.028 ± 0.002	0.026 ± 0.001	0.042	0.014	0.007	0.264		0.290
2	36.8 ± 5.8	51.1 ± 3.6	74.1 ± 2.0	0.164 ± 0.003	0.145 ± 0.002	0.100 ± 0.002	0.093 ± 0.000	0.076 ± 0.000	0.065 ± 0.000	0.029 ± 0.002	0.027 ± 0.002	0.027 ± 0.001	0.042	0.042	0.008	0.264		0.281
3	36.8 ± 5.8	64.4 ± 1.5	78.2 ± 3.1	0.166	0.138	0.111	0.093	0.072	0.072	0.030	0.029	0.029	0.042	0.036	0.010	0.264		0.281
4	39.3 ± 2.2	73.0 ± 3.3	64.9 ± 2.3	0.162	0.085	0.069	0.071	0.040	0.034	0.033	0.030	0.027	0.058	0.015	0.009			
5	48.0 ± 3.7	75.0 ± 2.5	66.7 ± 3.6	0.151	0.087	0.067	0.067	0.032	0.033	0.030	0.030	0.026	0.054	0.024	0.009			
6	36.9 ± 3.2	40.5 ± 4.9	47.6 ± 3.1	0.165 ± 0.002	0.163 ± 0.003	0.165 ± 0.002	0.094 ± 0.000	0.092 ± 0.000	0.095 ± 0.001	0.028 ± 0.002	0.028 ± 0.002	0.029 ± 0.002	0.043 ± 0.000	0.042 ± 0.001	0.040 ± 0.001	0.271		0.271
7	36.9 ±3.2	37.6 ± 4.8	40.2 ± 6.5															
8	36.8 ± 5.8	28.6 ± 5.7	34.2 ± 2.2	0.164 ± 0.003	0.164 ± 0.002	0.128 ± 0.001	0.093 ± 0.000	0.090 ± 0.001	0.091 ± 0.001	0.029 ± 0.002	0.027 ± 0.004	0.030 ± 0.001	0.042	0.047	0.007	0.264		0.271
9	42.2 ± 4.3	77.1 ± 4.0	80.3 ± 1.8	0.148	0.104	0.100	0.087	0.068	0.069	0.029	0.028	0.024	0.032	0.008	0.007	0.322		0.336
10	42.2 ± 4.3	80.8 ± 1.6	80.7 ± 1.4	0.148	0.102	0.091	0.087	0.065	0.060	0.029	0.028	0.024	0.032	0.009	0.007	0.322		0.339
11	43.9 ± 5.3	68.1 ± 1.0	63.0 ± 1.8	0.223	0.157	0.151	0.119	0.078	0.076	0.035	0.032	0.030	0.069	0.048	0.045	0.239	0.240	0.241
12	43.9 ± 5.3	69.0 ± 2.4	69.8 ± 1.4	0.223	0.151	0.147	0.119	0.074	0.072	0.035	0.031	0.029	0.069	0.047	0.046	0.239	0.239	0.246
13	43.9 ± 5.3	72.8 ± 2.5	60.9 ± 2.4	0.223	0.163	0.158	0.119	0.074	0.072	0.035	0.031	0.029	0.069	0.058	0.058	0.239	0.239	0.236
14																		
15																		
16																		
17																		
18																		
19																		
20																		

448 **(a)**

449

450

451

Experiment no.	Total protein analysis (Lowry method)			Total lipid and fatty acid analysis														
	Total protein content (mg / mg biomass)			Total lipid content (mg / mg biomass)			Total FAME content (mg / mg biomass)			C16:0 FAME content (mg / mg biomass)			C16:1 FAME content (mg / mg biomass)			C20:5 FAME content (mg / mg biomass)		
	duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)		
	0	24	72	0	24	72	0	24	72	0	24	72	0	24	72	0	24	72
1				0.288 ± 0.024	0.291	0.275 ± 0.021	0.107 ± 0.002	0.109 ± 0.005	0.165 ± 0.001	0.029 ± 0.000	0.029 ± 0.001	0.042 ± 0.000	0.029 ± 0.000	0.028 ± 0.000	0.041 ± 0.000	0.030 ± 0.002	0.033 ± 0.003	0.052 ± 0.001
2																		
3																		
4																		
5																		
6																		
7																		
8																		
9																		
10				0.252	0.297	0.272 ± 0.012	0.105 ± 0.008	0.144 ± 0.002	0.111 ± 0.002	0.024 ± 0.003	0.031 ± 0.002	0.023 ± 0.002	0.030 ± 0.001	0.038 ± 0.001	0.029 ± 0.001	0.034 ± 0.001	0.054 ± 0.001	0.042 ± 0.002
11																		
12				0.344	0.326	0.342 ± 0.027	0.185 ± 0.003	0.149 ± 0.003	0.201 ± 0.008	0.057 ± 0.000	0.046 ± 0.000	0.066 ± 0.002	0.046 ± 0.001	0.036 ± 0.001	0.051 ± 0.002	0.044 ± 0.001	0.036 ± 0.001	0.042 ± 0.002
13																		
14	0.183 ± 0.005	0.124 ± 0.004	0.178 ± 0.007															
15	0.132 ± 0.006	0.114 ± 0.004	0.093 ± 0.005															
16	0.115 ± 0.001	0.127 ± 0.007	0.105 ± 0.004															
17																		
18																		
19																		
20																		

452 **(b)**

Experiment no.	Fermentation products analysis																	
	Yield of identifiable fermentation products (mg / mg biomass)			Yield of succinic acid (mg / mg biomass)			Yield of lactic acid (mg / mg biomass)			Yield of fumaric acid (mg / mg biomass)			Yield of acetic acid (mg/ mg biomass)			Yield of propionic acid (mg / mg biomass)		
	duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)		
	0	24	72	0	24	72	0	24	72	0	24	72	0	24	72	0	24	72
1	0.0029	0.0299	0.0680	0.0008	0.0159	0.0189	0.0000	0.0057	0.0012	0.0012	0.0040	0.0075	0.0000	0.0033	0.0160	0.0004	0.0005	0.0234
2	0.0029	0.0255	0.0571	0.0008	0.0206	0.0235	0.0000	0.0001	0.0034	0.0012	0.0009	0.0028	0.0000	0.0013	0.0092	0.0004	0.0020	0.0173
3	0.0029	0.0289	0.0493	0.0008	0.0189	0.0195	0.0000	0.0015	0.0044	0.0012	0.0046	0.0062	0.0000	0.0019	0.0035	0.0004	0.0009	0.0132
4	0.0071	0.0411	0.0633	0.0030	0.0114	0.0113	0.0001	0.0174	0.0260	0.0015	0.0056	0.0137	0.0006	0.0058	0.0074	0.0009	0.0001	0.0043
5	0.0102	0.0513	0.0817	0.0020	0.0305	0.0335	0.0000	0.0082	0.0289	0.0042	0.0081	0.0089	0.0004	0.0020	0.0025	0.0016	0.0016	0.0051
6	0.0033	0.0064	0.0059	0.0011	0.0009	0.0009	0.0000	0.0000	0.0000	0.0015	0.0030	0.0028	0.0000	0.0007	0.0005	0.0003	0.0014	0.0006
7	0.0033	0.0052	0.0087	0.0011	0.0009	0.0009	0.0000	0.0000	0.0004	0.0015	0.0022	0.0027	0.0000	0.0005	0.0014	0.0003	0.0013	0.0023
8	0.0029	0.0135	0.0428	0.0008	0.0033	0.0016	0.0000	0.0060	0.0218	0.0012	0.0017	0.0060	0.0000	0.0004	0.0107	0.0004	0.0006	0.0014
9	0.0031	0.0258	0.0396	0.0006	0.0040	0.0023	0.0000	0.0072	0.0022	0.0013	0.0022	0.0089	0.0002	0.0115	0.0093	0.0006	0.0004	0.0158
10	0.0031	0.0252	0.0431	0.0006	0.0056	0.0032	0.0000	0.0015	0.0002	0.0013	0.0027	0.0046	0.0002	0.0129	0.0284	0.0006	0.0021	0.0063
11	0.0030	0.0406	0.0756	0.0016	0.0164	0.0166	0.0000	0.0171	0.0169	0.0000	0.0037	0.0317	0.0002	0.0016	0.0055	0.0003	0.0001	0.0019
12	0.0030	0.0395	0.0763	0.0016	0.0153	0.0172	0.0000	0.0165	0.0181	0.0000	0.0048	0.0324	0.0002	0.0015	0.0042	0.0003	0.0001	0.0021
13	0.0030	0.0374	0.0514	0.0016	0.0153	0.0175	0.0000	0.0166	0.0188	0.0000	0.0033	0.0084	0.0002	0.0011	0.0024	0.0003	0.0001	0.0025
14																		
15																		
16																		
17																		
18																		
19																		
20																		

453 **(c)**

Experiment no.	Cell wall analysis with Transmission Electron Microscopy						Cellulose analysis			Cell viability analysis					
	Thickness of cellulose layer (nm)			Thickness of algaenan layer (nm)			Total cellulosic glucose content (mg / mg biomass)			Proportion of signal attributed to whole cells (% of total signal)			Proportion of non-viable cells (% of available cells)		
	duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)		
	0	24	72	0	24	72	0	24	72	0	24	72	0	24	72
1	23.2 ± 5.1	9.0 ± 3.0	9.1 ± 4.6	5.6 ± 0.9	5.1 ± 0.9	4.9 ± 0.8				60.2	33.3	27.0	7.7	27.1	65.0
2										60.2	47.7	24.2	7.7	6.7	56.6
3	23.2 ± 5.1	12.0 ± 2.8	9.9 ± 2.0	5.6 ± 0.9	5.3 ± 0.6	5.0 ± 1.0				60.2	40.8	26.0	7.7	15.1	44.4
4										73.3	58.5	75.5	2.8	31.0	56.2
5										82.8	78.5	78.4	2.5	7.2	42.6
6	22.4 ± 5.1	20.9 ± 5.5	20.4 ± 6.2	4.6 ± 0.8	4.6 ± 0.7	5.2 ± 0.8				54.4	49.2	43.9	5.5	9.4	18.6
7										54.4	64.2	62.8	5.5	5.9	12.2
8	23.2 ± 5.1	20.0 ± 2.3	19.3 ± 3.2	5.6 ± 0.9	5.0 ± 0.7	5.4 ± 0.6				60.2	39.3	29.5	7.7	12.8	66.7
9										71.5	79.5	68.9	11.4	75.6	86.7
10	21.6 ± 6.1	9.6 ± 2.9	9.2 ± 3.9	5.0 ± 0.7	5.3 ± 1.1	5.4 ± 0.9				71.5	69.4	71.2	11.4	30.2	86.9
11							0.093	0.065	0.057	67.4	40.3	22.5	5.7	7.3	52.2
12	36.6 ± 7.7	18.4 ± 5.4	17.3 ± 4.0	5.4 ± 0.9	5.4 ± 1.4	5.1 ± 0.6	0.093	0.063	0.058	67.4	38.1	24.9	5.7	8.5	63.7
13							0.093	0.060	0.058	67.4	41.2	20.0	5.7	9.3	75.6
14															
15															
16															
17															
18															
19															
20															

454

(d)

Experiment no.	Cell rupture analysis									Lipid extraction with hexane								
	Extent of cell rupture after HPH (% of available cells)			Extent of cell rupture after alkaline treatment (% of available cells)			Extent of cell rupture after acidic treatment (% of available cells)			Lipid yield of HPH downstream pathway (wt% of total lipid)			Lipid yield of alkaline treatment downstream pathway (wt% of total lipid)			Lipid yield of acidic treatment downstream pathway (wt% of total lipid)		
	duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)			duration of incubation treatment (h)		
	0	24	72	0	24	72	0	24	72	0	24	72	0	24	72	0	24	72
1																		
2																		
3																		
4																		
5																		
6																		
7																		
8																		
9																		
10																		
11																		
12																		
13																		
14																		
15																		
16																		
17	25.5 ± 9.0	91.7 ± 2.6																
18	36.6 ± 13.4	90.2 ± 2.0		14.1 ± 9.5	79.8 ± 5.8					28.3 ± 0.6	51.3 ± 5.6		5.4 ± 6.7	33.1 ± 8.4				
19	20.3 ± 5.9	49.4 ± 10.6								31.7 ± 15.9	50.0 ± 17.0		10.7 ± 11.7	22.8 ± 2.5				
20	47.3 ± 14.1	88.4 ± 2.7		30.2 ± 8.8	73.7 ± 3.3		20.9 ± 16.5	42.8 ± 7.5		26.3 ± 10.5	41.1 ± 8.9		4.4 ± 1.7	23.3 ± 3.1		5.0 ± 4.4	19.6 ± 7.5	

455

(e)