Lipid Productivity and Cell Wall Ultrastructure of Six Strains of *Nannochloropsis*: Implications for Biofuel Production and Downstream Processing.

Tracey A. Beacham [a], Claire Bradley[a], Daniel A. White[a], Peter Bond,[b] & Sohail T. Ali[a]#

Plymouth Marine Laboratory, Prospect Place, Plymouth, PL1 5DH, UK 01752 633100[a,]

Plymouth Electron Microscopy Centre, Plymouth University, Drake Circus, Plymouth, PL4 8AA, UK [b]

Running Head: Cell Wall Ultra-Structure of *Nannochloropsis*

#Address correspondence to Sohail T. Ali, stal@pml.ac.uk,

Abstract

Microalgae are generating considerable interest for third generation biodiesel production. However, appropriate strain selection is proving challenging due to the significant variation in cellular physiology, metabolic potential and genetics observed even amongst strains deemed morphologically similar. Six strains of *Nannochloropsis* from the CCAP culture collection were assessed for their lipid productivity and cellular structure, as proxies for oil production and harvesting ease, to assess their suitability as biodiesel production platforms. Differences in growth rate and lipid accumulation across the strains were observed. *N*. *oculata* strain 849/7 showed significantly reduced doubling time compared to *N*. *salina* strain 849/3, whilst *N. oceanica* 849/10 produced the highest lipid content. In addition the six strains could be differentiated in to 3 distinct classes based on their cell wall thickness, which varied across the strains from 63-119 nm and which is independent of both species and geographical isolation location. The importance of these variations in ultrastructure and physiology for biodiesel production is discussed.

Key words

Nannochloropsis, Microalgae; fatty acid; lipid; ultrastructure; biofuels

1.1 Introduction

There has been considerable interest in microalgae as a potential alternative, low carbon, renewable source of bioenergy and chemical feedstock [1, 2]. In particular, the need for alternative sources of liquid transportation fuels to replace the over-reliance on diminishing sources of mineral oil is crucial to economic stability and development. This is compounded by the need to reduce CO_2 emissions to mitigate global climate change resulting from increasing levels of atmospheric CO_2. In 2011 global energy demand rose 2.5% with 33% of all consumption derived from oil [3]. Research aimed at the development of carbon neutral liquid, gaseous and solid sources of renewable energy has in recent years started to move away from biofuels primarily derived from food crops and oily seeds which impact negatively on both food and water security and contribute to the destruction of the world's forests [4,5,6]. Biofuels from waste cooking oil and animal fat have grown in production but cannot satisfy the current demand for transport fuel [4] and have been shown to make a limited contribution to climate change mitigation [7].

Microalgae show promise as a high impact source of biomass for biofuel production. These photosynthetic microorganisms have low input requirements for light and nutrition whilst producing large amounts of biomass over short periods of time, including processing lipids for biofuels and valuable co-products [6].

When compared to current approaches with terrestrial crops, algae have much higher predicted energy yields per area [5, 8] and whilst algae production is known to consume

more water than other biofuel sources produced from terrestrial growth [8], use of marine species would not impact on decreasing fresh water supplies.

The economic and technical barriers involved in micro algal derived biofuel production have begun to be addressed in recent years with heavy investment from both government and industrial sponsors [5, 9]. These research programs have seen technology improvements enabling higher biomass / hectare output combined with reduced cost of dewatering, extraction and refining. Despite these technical advances algal derived biofuels remain uncompetitive with present day fossil fuels [5]. It is well-known that many algae accumulate triacylglycerol in large quantities during the stationary phase of growth [4, 10], however, to be commercially viable production requires a semi-continuous culture maintained at mid-logarithmic phase suitable for non-stop culture.

Traditional cross breeding in plants is an effective method for improving the yield of oil seed crops, yet such an approach with algae is not feasible. Whilst bioengineering offers the possibilities for improving lipid accumulation and a raft of other important traits such as thermotolerance and photosynthetic efficiency, many species of algae remain resistant to the standard molecular techniques available. For these reasons optimal microalga strain selection is critical to maximise biofuel production.

An initial analysis of several microalgal species suggested that *Nannochloropsis* sp. showed the most promise as a potential feedstock for biofuel production. The *Nannochloropsis* genus is a diverse collection of microalgae comprising 6 species containing several sub strains within this. In fact, a recent genomic analysis of 6

species of *Nannochloropsis* has revealed considerable genetic diversity amongst species despite strong conservation of 18S rRNA genes [11]. To assess the potential of *Nannochloropsis* as a biofuel producing strain and to develop a better understanding of the physiological differences which may exist between the different *Nannochloropsis* species a total of four *Nannochloropsis* species representing six different strains were analysed for fatty acid composition and lipid productivity. Since these species are also known to be very robust and refractory to cellular disruption [12, 13, 14] a detail that could impact on the ease and efficiency of oil extraction, an ultra-structural assessment of the cell wall of each species using electron microscopy was performed. Considerable variations in both lipid accumulation and cell wall thickness were observed and the implication of these observations for biofuel production and downstream processing of algal biomass is discussed.

2. Methods

2.1 Strains.

Algae strains were obtained from the Culture Collection of Algae and Protozoa (Scottish Association for Marine Science, Oban, Scotland, U.K.). Cultures assessed in this investigation: *Nannochloropsis salina* (CCAP 849/3 isolated from Skate Point, Isle of Cumbrae, Scotland and CCAP 849/6 isolated from Great South Bay, Long Island, New York, USA), *N. gaditana* (CCAP 849/5 isolated from Cadiz Bay, Cadiz, Spain), *N.oculata* (CCAP 849/1 from Skate Point, Isle of Cumbrae, Scotland and CCAP 849/7

isolated from Lake of Tunis, Tunisia), *N. oceanica* (CCAP 849/10; no geographical data).

2.2 Culture conditions.

Stock cultures were maintained under batch culture conditions (1L) in F/2 medium [15] and sub-cultured on a weekly basis. Experimental cultures (250 ml in 1 L flasks) were maintained under 100 µmols photons m^2 sec^{-1} irradiance on a 16 h: 8 h light: dark cycle at 25 ºC (±1^0C) and agitated daily but provided no additional aeration. Cultures for comparison of 90% salinity (~30.6ppt) and 10% salinity (~3.4ppt) acclimated cells were grown in 2 L growth vessels and bubbled with air under the same light conditions. All experimental cultures were inoculated at a density of 1 x 10^5 cells ml^{-1} and maintained without sub-culturing into stationary growth phase. Samples were removed and analysed for cell growth, cell wall phenotype, and cellular lipids analysed. Culture growth rates and cell densities were monitored as described below.

2.3 Growth rate determination.

Culture density was determined via light microscope cell enumeration in a haemocytometer following staining with Lugols iodine solution (2 %). Specific growth rates (K) were calculated according to the following equation:

$$K = \ln(N2/N1) / (t2-t1)$$

where N2 and N1 are the total cells mL^{-1} at time point (t2) and time point (t1) respectively, and where t2>t1.

2.4 Lipid analyses.

For each strain, at each sampling point 5 x 50ml samples were subject to lipid analysis. Fatty acid concentrations and profiles in microalgal cells were determined post conversion to fatty acid methyl esters (FAMEs) using GC-MS (Agilent 7890A GC and 5975C inert MSD, Agilent Technologies Ltd., Edinburgh, UK). Culture samples were centrifuged (10,000 × *g*), washed in distilled water and resulting pellets lyophilised. Nonadecanoic acid (C19:0) was added as an internal standard and cellular fatty acids were converted directly to FAMEs by adding 1mL of transesterification mix (95:5 v/v 3 N methanolic HCl; 2,2-dimethoxypropane) followed by incubation at 90 ºC for 1h. After cooling, FAMEs were recovered by addition of 1 % w/v NaCl solution (1 mL) and *n*-hexane (1 mL) followed by vortexing. The upper hexane layer was injected directly onto the GC-MS system as previously described in White *et al.* [16].

2.5 Electron microscopy.

Exponentially growing cells harvested 5 days after culture initiation were washed in fresh F/2 medium and re-suspended in fixative (2.5 % glutaraldehyde in PBS) at an approximate cell density of 1 x 10^9 cells ml^{-1} for 4 hours at 4 °C. Fixed cells were washed twice in PBS and then immobilised in 2 % low melting point agarose. The agarose pellet (just the area with cells) was cut into small blocks (1x1x1 mm) which were post-fixed in 1 % osmium tetroxide in PBS, dehydrated in an alcohol series and embedded in Agar Low Viscosity Resin. Ultra-thin sections were cut with a Leica EM UC7 ultra microtome, stained with uranyl acetate and lead citrate, and examined in a JEOL 1200EX transmission electron microscope. Analysis of the cell wall thickness was performed using ImageJ [17] with individual cells measured in 5 separate places and a total of 50 individual cells per strain assessed.

2.6 Statistical analysis

Data was assessed for normality and then subject to ANOVA and 2-sample T testing. P values of less than 0.05 were considered to be significant.

3. Results and Discussion

The growth rate has a major impact on the maximum productivity that a strain can achieve, and also impacts on any lag time in culture recovery post-harvest, especially where a culture has been allowed to enter stationary phase. The growth properties of the six strains grown in batch culture over 27 days is summarised in Figure 1. All strains demonstrated a substantial biomass production before entering stationary phase with an average maximum cell density across all strains of 32 million cell mL^{-1} (Figure 1).

Both *N. oculata* strains (849/1 and 849/7) achieved significantly higher cell densities than the other species assessed (p=<0.05) with strain 849/1 achieving a 38% higher cell density than the least prolific strain *N. salina* (849/6) (Table 1). The average doubling time during the initial growth phase was fairly consistent across the *N. salina*, *N. gaditana* and *N. oceanica* strains (40-46 hours). *N*. *oculata* strain 849/7, however, showed significantly reduced doubling time of 35.3 hours compared to *N*. *salina* strain 849/3 with a doubling time of 45.9 hours (p=0.042).

Lipid content steadily increases during the growth phase [4, 10] and it can vary significantly between the early exponential and stationary phases of growth. To be

commercially viable however, algae will need to be maintained under continuous culture conditions without any lag post-harvest, and as such lipid analysis to measure the FAME content and composition was performed during the exponential growth phase (Day 5). Total FAME content (Figure 2) varied widely both across the species from 3.5% in *N*. *oculata* strain 849/1 to 14.7% in *N*. *oceanica* strain 849/10, and within sub-strains of the same species with the second *N. oculata* strain (849/7) achieving 8.86% total FAME - more than double that of 849/1 (P=>0.05).

Diversity of fatty acid saturation was relatively low and with the exception of 849/1, all strains showed a preference for saturated and monounsaturated fatty acid production over the long chain polyunsaturated fatty acids. Strain 849/1 showed an equal FAME content of SFAs, MUFAs and PUFAs (Fig. 2). The lower level of oil accumulation combined with the high polyunsaturated nature of the FAME components make this strain particularly unsuited to biofuel production.

N. *oceanica* strain 849/10 had both the highest total FAME accumulation and the lowest overall level of unsaturation (Fig. 2). *N. salina* strain 849/3 and *N. oculata* strain 849/7 both had high levels of FAME and though the level of unsaturation was elevated ($p<0.05$) in comparison to 849/10, the PUFA content of these two strains was not considered to be incompatible as biofuel feedstock. In addition, analysis of the individual fatty acid profiles (Table 2) showed 849/3 and 849/7 produced a significantly higher ratio of palmitic acid (16:1) to oleic acid (18:1) compared to 849/10 and 849/6. With winter and summer blends of biodiesel having differing requirements in terms of viscosity and flash point the availability of strains with different FAME profiles raises the

possibility of biofuel production where the algal strain is alternated depending on the fuel requirements.

Cell wall thickness is an important trait to be considered, since it affects not only the carbon budget of the organism, ease of downstream extraction of the oil and processing of residual biomass [18] but is also a major barrier to bioengineering. The *Nannochloropsis* strains were subjected to TEM analysis. Figure 3 shows a representative image from each strain and the average cell size and cell wall thickness. Inter and intraspecies cell size (Figure 3A) varied significantly from 2.31 – 2.77μm with *Nannochloropsis gaditana* 849/5 attaining a size significantly larger than all the other species ($p=<0.05$). No significant difference in cell size was observed between the *N. salina* sub strains*, but the N. oculata* strains varied from 2.36μm (849/1) to 2.59μm (849/7) $p=<0.05$.

Cell wall thickness varied widely both between the 4 different species and surprisingly between the sub strains of the same species. The strains fell into three statistically distinct groupings as summarised in Figure 3b, with very thick (849/1, 849/6, 849/10) in the range 107-119nm, thick (849/5, 849/7) in the range 82-90nm or thin cell walls (849/3) in the range 63-69nm. *N*. *salina* strains were most divergent with 849/3 having the thinnest cell wall of all the strains, measuring on average 66nm, and 849/6 having one of the thickest cell walls, measuring on average 108 nm.

There appears to be no geographical correlation between cell wall thickness and original isolation sites and it is interesting to note that 849/3 (thin cell walls) and 849/1 (very thick cell walls) were both isolated from the same location at Skate Point, Isle of

Cumbrae, Scotland. In addition, since all the strains have been maintained for several years under the same laboratory conditions it would suggest that the observed differences in cell wall thickness are not phenotypic fluctuations due to some transient environmental condition but are a distinct genetic trait of each strain. For an alga to be integrated into a commercially viable industrial scale process it needs to demonstrate a significant level of robustness in its tolerance to changes in growth conditions such as salinity and temperature. This is especially so where waste-water or growth medium recycling is likely to feature which may cause significant variations in the chemical composition and salinity of the medium.

The effect of low salt conditions on *Nannohcloropsis* growth and the effect of salinity on cell ultrastructure were determined. *N. salina* strain 849/3 was acclimated from standard 90% seawater F/2 medium (approximately 3.1% total salt) to a 10% seawater F/2 medium (approximately 0.35% total salt) stepwise in 4 increments (70%, 50%, 30%, 10% seawater) over a period of two months. Greater dilution of the seawater at each step caused the culture to die.

Changes in lipid productivity, growth and ultra-structure were assessed as before. Reduced saline growth conditions caused a thickening of the cell wall of around 20% in response to the elevated osmotic potential (Table 3). A representative TEM image of cells grown under each condition is given in Figure 4 and the ultra-structure change is clearly visible. Growth analysis showed no difference in doubling time or maximum cell density.

Comparative analysis of lipid productivity and quality (Table 3 and Figure 4A) indicated little difference in total FAME content between the 10% and 90% salinity cultures with only a slight though significant increase in the 90% salinity cells during mid-exponential growth phase ($p=<0.05$). No significant changes in the fatty acid profile were observed between the cultures in the exponential phase of growth, however, during the stationary phase the level of PUFAs was reduced when cells were grown at 90% salinity. Long (16-18C) and very long chain (>18C) PUFAs are primarily used by micro algae in cell wall structure, and are involved in membrane fluidity maintenance, plastid structure and function and are often substrates for lipoxygenase. The oxylipins produced have roles in signalling both wound-healing and stress and many have roles in innate immunity with antimicrobial and anti-fungal properties [19, 20, 21, 22]. We consider that the difference the level of saturation and the elevation in the long and very long chain fatty acid between the 10 and 90% salinity cells (Table 3) and elevated requirement for these structurally important fatty acids during stationary phase is due to both the increased cell wall thickness and the presence of altered consortia of symbiotic and competitive microorganisms in the reduced saline growth environment.

These results indicate that moderate salinity changes (up to 20%) in culture are unlikely to hinder the culture growth. The shift from 90% salinity to 10% salinity and corresponding increase in cell wall thickness did however have small negative impact on lipid productivity during exponential growth (the optimal growth phase for lipid production) and supports other work indicating that diverting carbon from cell wall production towards lipid production may be beneficial for lipid-based biofuel production. Increased cell wall thickness due to growth under decreased salinity may provide

increased residual biomass for either anaerobic digestion [23] or conversion to another bulk chemical feedstock through, for example, pyrolysis. Such an approach would result in the need for a more intense method for cell lysis and an increased use of freshwater, negating a major reason for developing marine strains for feedstock production.

Choice of the species to be used in microalgal derived biofuel is therefore vital to ensure maximum productivity. Modern photobioreactor (PBR) technologies lend themselves to continuous or semi continuous biomass production maintaining the algal culture in the growth phase. This type of culturing with an algal species such as *Nannochloropsis salina* 849/3, which is capable of accumulating relatively high amounts of lipid during mid-to-late growth phase, could improve the productivity over traditional stationary phase harvesting. Based on the data shown in Figure 4A and Table 3 for cells grown under 90% salinity conditions, assuming 45% lipid content (10pg/cell) and a maximum cell density of $1.17x10^{8}$ cells ml^{-1} , yields of around 468g oil would be obtained from a 400L scale culture after 25 days (harvesting cells during stationary phase). This compares to a semi-continuous harvesting of 20% volume daily of an actively growing culture at 17.5% lipid content (3.88pg/cell) and a cell density of $9.8x10^{7}$million cells ml^{-1} , which would yield around 30g oil per day or 762g oil over a 25 day period. This equates to a productivity increase of approximately 39%.

Quin *et al*. [24] have developed a 174,000 L outdoor photobioreactor where the major variable is light. Of particular interest is their use of *Nannochloropsis oculata* CCMP 525 which is the same as CCAP 849/1 used in this study, so allowing a direct comparison of productivity. Quin *et al*. report an average annual productivity of 0.15 g L^{-1} d^{-1} with the highest productivity recorded in the summer months reaching 0.37 g L^{-1}

d^{-1} which is comparable to this study (0.432 $g^{-1}/L^{-1}/d^{-1}$ when grown in a small scale indoor PBR as described above). This suggests that similar productivity is observed under laboratory conditions as in outdoor systems and any shortfall in productivity would be due to seasonal variations rather than issues of large scale cultivation. When considering that agricultural growth is dominated by seasonal cultivation, seasonal variation in both light and temperature should not be seen as a negative factor in the development of large-scale outdoor cultivation and continued production in the winter months considered an advantage. However, not only can the total lipid content in outdoor facilities vary by up to 50% over the year but this variation can affect the fatty acid profile so altering the quality of the final product [25]. Reducing the environmental variability in outdoor cultivation is thus desirable but the added energy (and carbon) costs must be carefully considered.

The present study demonstrates the wide physiological variation across *Nannochloropsis* species and strains with differences observed both in lipid biosynthesis and cell ultrastructure. Several of the *Nannochloropsis* strains assessed demonstrated high lipid accumulation during the growth phase combined with fast growth rates and an ability to grow to high cell densities. For example, *N. oceanica* strain 849/10 combines both high oil production and low levels of polyunsaturation with substantial levels of cell proliferation accumulating at high cell densities. However this strain also has one of the thickest cell walls of all the strains tested which could affect the efficiency of downstream processing. In contrast, the good growth and metabolic characteristics coupled with a much thinner cell wall found for *Nannochloropsis salina* strain 849/3 may make it a better prospect for biofuel production overall if downstream

303 processing is found to be better for this strain. The increased cell wall thickness this
304 strain exhibited when exposed to decreased salinity also suggests that the composition
305 of the growth medium (and possibly CO_2 concentration) could play an important role in
306 algal growth beyond influencing the quantity and quality of lipids produced by a given
307 strain with unexpected implications for downstream harvesting and processing of the
308 resultant biomass.

309 Thus, a better understanding of the cell biology and physiology of microalgae beyond
310 simply lipid content is necessary if microalgae-derived biofuels are to become an
311 economic reality. This study further supports the diversity of *Nannochloropsis* species
312 as demonstrated by the recent genomic sequencing [11, 26, 27] on 6 *Nannochloropsis*
313 species and suggests that rDNA sequencing is an inadequate marker in isolation for
314 algal species classification.

315

316 **4. Conclusion.**

317 Investigation of 6 individual *Nannochloropsis* species including phenotypic assessment
318 of growth and cell wall thickness as well as GC-MS analysis of fatty acid content and
319 composition showed that *Nannochloropsis salina* strain 849/3 has the best combination
320 of the desired characteristics essential to ensure the high productivity of biomass
321 required for biofuel feedstock. Further, we have demonstrated that the robustness of *N.*
322 *salina* (849/3) to changes in salinity will allow for flexibility in growth media compatible
323 with waste water recycling and also makes this strain particularly suitable for

bioengineering. The relatively narrow cell wall thickness of this strain should also aid in the ease of cell rupture for oil extraction, thus reducing downstream processing costs.

5. References:

[1] A. Demirbas, M.F. Demirbas, Importance of algae oil as a source of biodiesel, Energ Convers and Manage, 52 (2011) 163–170

[2] B. Liu, C. Benning, Lipid metabolism in microalgae distinguishes itself, Curr. Opin. Biotechnol. 24 (2013) 300-309 DOI: 10.1016/j.copbio.2012.08.008

[3] BP, Statistical review of world energy, BP Worldwide, (2012)

[4] Y. Chisti Biodiesel from microalgae. Biotechnol Adv, 25 (2007) 294-306

[5] N.H. Norsker, M.J Barbosa, M.H.Vermuë, R.H. Wijffels, Microalgal production - A close look at the economics. Biotechnol. Adv, 29 (2011) 24–27

[6] L. Brennan, P. Owende, Biofuels from microalgae - A review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Energ Rev, 14 (2010) 557-577, doi:10.1016/j.rser.2009.10.009

[7] T.M. Mata, A.A Martins, Caetano, S. Nidia, Microalgae for biodiesel production and other applications: A review, Renew Sust Energ Rev, 14 (2010) 217-232

[8] A.F. Clarens, P. Eleazer, E.P. Resurreccion, M.A. White, L.M. Colosi, Environmental life cycle comparison of algae to other bioenergy feedstocks. Environ Sci Technol. 44 (2010) 1813–1819

[9] P.T. Pienkos, A. Darzins, The promise and challenges of microalgal-derived biofuels. Biofuel Bioprod Bior, 3 (2009) 431–440. doi: 10.1002/bbb.159

[10] P. Spolaore, C. Joannis-Cassan, E. Duran, A. Isambert, Commercial applications of microalgae. J Biosci Bioeng, 101 (2006) 87–96

[11] D. Wang, K. Ning, J. Li, *et al. Nannochloropsis* genomes reveal evolution of microalgal oleaginous traits, PLOS genet, (2014) 10 (1):e1004094. Doi:10.1371/journal.pgen.1004094

[12] J.R. McMillan, I.A.Watson, M. Ali, W. Jaafar Evaluation and comparison of algal cell disruption methods: Microwave, waterbath, blender, ultrasonic and laser treatment, Appl Energ, 103 (2013) 128–134

[13] S. Schwede, A. Kowalczyk, M. Gerber, R. Span, Influence of different cell disruption techniques on mono digestion of algal biomass. Proceedings of the World Renewable Energy Congress, Linköping, Sweden (2011)

[14] R.D. Long, E. Abdelkader, Mixed-polarity azeotropic solvents for efficient extraction of lipids from *Nannochloropsis* microalgae, Am J Biochem Biotechnol, 7 (2011) 70-73

[15] R.R.L. Guillard, Culture of phytoplankton for feeding marine invertebrates. *In* Smith WL, Chanley MH (ed), Culture of marine invertebrate animals. Plenum Press, New York pp. (1975) 26-60

[16] D.A. White, A. Pagarette, P. Rooks, S.T. Ali The effect of sodium bicarbonate supplementation on growth and biochemical composition of marine microalgaecultures, J. Appl. Phycol, 25 (2013) 153-165

[17] M.D Abramoff, P.J. Magalhaes, S.J. Ram, Image Processing with ImageJ, Biophotonics Internat., 11 (2004) 36-42

[18] S. Schwede, Z.U. Rehman, M. Gerber, C. Theiss, R. Span, Effects of thermal pretreatment on anaerobic digestion of *Nannochloropsis salina* biomass, Bioresour Technol., 143 (2013) 505-11. doi: 10.1016/j.biortech.2013.06.043

[19] G.A. Thompson Lipids and membrane function in green algae, Biochim Biophys Acta., 1302 (1996)7-45

[20] H. Porta, M. Rocha-Sosa Plant lipoxygenases: physiological and molecular features, Plant Physiol., 130 (2002) 15–21

[21] I.A. Guschina, J.L. Harwood Lipids and lipid metabolism in eukaryotic algae, Prog Lipid Res., 45 (2006) 160–186

[22] K. Bouarab, F. Adas, E. Gaquerel, B. Kloareg, J.P. Salau, P. Potin, The Innate immunity of a marine red alga involves oxylipins from both the eicosanoid and octadecanoid pathways, Plant Physiol., 135 (2004) 1838–1848

[23] B. Sialve, N. Bernet, O. Bernard, Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable, Biotechnol Adv., 27 (2009) 409–416.

[24] J.C. Quinn, T.Yates, N. Douglas, K. Weyer, J. Butler, T.H. Bradley, P.J. Lammers, Nannochloropsis production metrics in a scalable outdoor photobioreactor for commercial applications. Bioresource Technology. 117 (2012) 164–171.

[25] M. Olofsson, T. Lamela, E. Nilsson, J. P. Bergé, V. del Pino, P. Uronen, C. Legrand. Seasonal Variation of Lipids and Fatty Acids of the Microalgae *Nannochloropsis oculata* Grown in Outdoor Large-Scale Photobioreactors. Energies, 5 (2012)1577-1592, doi:10.3390/en5051577

[26] A. Vieler, G. Wu, C.H. Tsai, B. Bullard, A.J. Cornish, et al. Genome, Functional Gene Annotation, and Nuclear Transformation of the Heterokont Oleaginous Alga *Nannochloropsis oceanica* CCMP1779. PLoS Genet 8 (2012): e1003064. doi:10.1371/journal.pgen.1003064.

[27] R. Radakovits, R.E. Jinkerson, S.I. Fuerstenberg, H.Tae, R.E. Settlage, J.L. Boore, M.C. Posewitz. Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropis gaditana*. Natture communicatitons (2012) DOI: 10.1038/ncomms1688.

Table 1. Average initial growth rate (doubling time) and the maximum cell density achieved ± SEM for 6 *Nannochloropsis* strains.

Strain	Average doubling time (hours)	Maximum cell density (cell/ml)
849/1	39.8 ± 1.1	$42 \times 10^6 \pm 2.4 \times 10^6$
849/3	45.9 ± 2.4	$28 \times 10^6 \pm 0.4 \times 10^6$
849/5	43.3 ± 2.8	$34 \times 10^6 \pm 1.1 \times 10^6$
849/6	40.0 ± 2.4	$26 \times 10^6 \pm 1.7 \times 10^6$
849/7	35.3 ± 2.3	$36 \times 10^6 \pm 1.2 \times 10^6$
849/10	40.6 ± 0.5	$30 \times 10^6 \pm 1.7 \times 10^6$

Table 2. Fatty acid profile of six *Nannochloropsis* strains during early exponential phase of growth. Data presented as percentage of total FAME content ± S.D.

Strain	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:3	C20:4	C20:5	Other
849/1	4.7±0.1	28.1±0.6	28.5±0.2	0.5±0.0	4.5±0.8	2.4±0.1	1.0±0.0	6.1±0.1	6.0±0.1	16.0±0.2	2.3±1.0
849/3	3.2±0.0	38.5±0.5	34.2±0.1	1.4±0.0	5.5±0.2	1.3±0.0	0.8±0.0	3.5±0.7	3.3±0.1	6.6±0.0	1.7±0.3
849/5	3.4±0.2	36.8±2.5	33.4±1.4	2.1±0.1	4.3±0.6	1.7±0.0	0.8±0.1	1.4±1.6	4.3±0.2	9.4±0.5	2.3±0.6
849/6	2.5±0.5	36.3±7.0	33.0±5.6	1.7±0.6	13.3±15	1.5±0.3	0.8±0.2	1.5±0.2	3.0±0.5	5.3±0.9	1.2±0.5
849/7	5.2±0.2	37.0±0.7	36.4±0.5	1.2±0.1	4.8±0.9	2.2±0.1	0.3±0.2	0.9±0.0	3.3±0.1	7.7±0.3	1.1±0.6
849/10	6.8±0.1	37.1±0.5	31.6±0.6	1.9±0.1	11.3±0.2	0.8±0.0	0.3±0.0	0.7±0.0	2.2±0.0	5.7±0.1	1.6±0.2

Table 3. Comparative analysis of the effect of salinity on important physiological parameters

	Mid exponential					Stationary			
Salinity	Cell wall thickness (nm)	Cell density (cells/ml)	SFA	MUFA	PUFA	Maximum Cell density (cells/ml)	SFA	MUFA	PUFA
90%	65.5 ± 2.8	9.8.E+07	39.4	35.0	25.6	1.17.E+08	41.1	40.8	18.1
10%	82.7 ± 2.6	9.5.E+07	40.1	34.4	25.5	1.21.E+08	31.1	45.4	23.5

Figure Legends

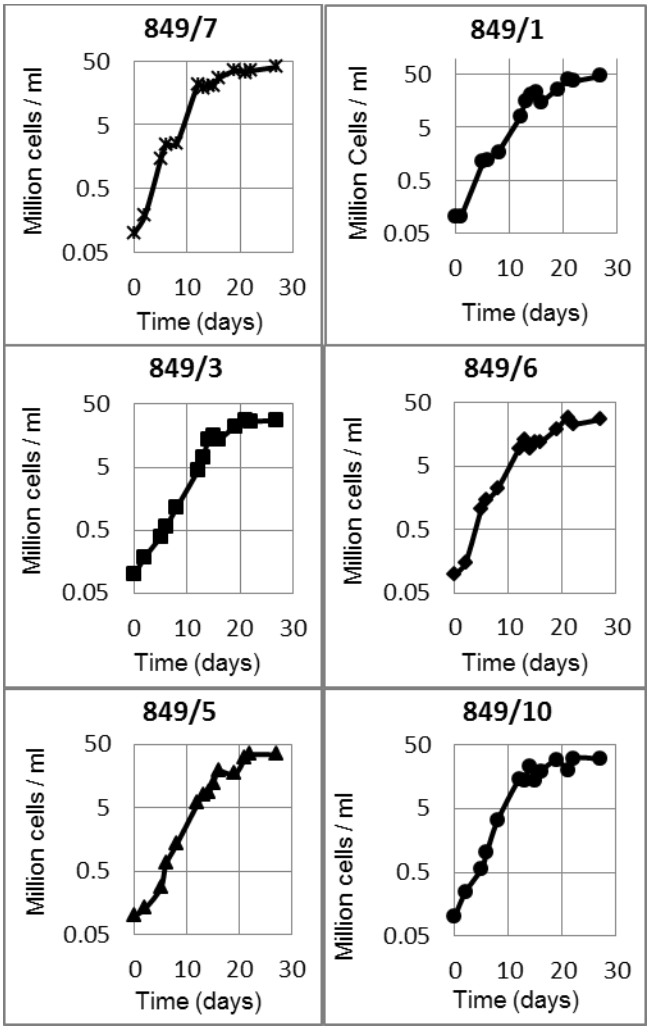
Figure 1. Comparison of population growth over 27 day period for 6 *Nannochloropsis* strains grown under batch culture conditions. Density is given as 10^6 cells ml^{-1}. Strains: 849/1 and 849/7 *Nannochloropsis oculata*, 849/3 and 849/6 *Nannochloropsis salina*, 849/5 *Nannochloropsis gaditana*, 849/10 *Nannochloropsis oceanica*.

Figure 2. Comparison of Total FAME content ± S.E.M and degree of fatty acid saturation in six *Nannochloropsis* strains. PUFA; Polyunsaturated fatty acids, MUFA; Monounsaturated fatty acids, SFA; saturated fatty acids

Figure 3. Comparison in cell wall thickness of six *Nannochloropsis* strains. (A) Cell wall thickness in tabular form ± SEM. Interval plot (B) provides a graphical representation of the data indicating the three statistically distinct size groupings ($p<0.05$). (C) Representative sample image of each strain, with the scale bar indicating 1 µm. Six *Nannochloropsis* strains were fixed and then subject to TEM analysis. The cell wall of individual cells was measured in 5 separate places and a total of 50 individual cells per strain were measured. CCMP strains: 849/1 and 849/7 (*Nannochloropsis oculata)*, 849/3 and 849/6 (*Nannochloropsis salina)*, 849/5 (*Nannochloropsis gaditana)*, 849/10 (*Nannochloropsis oceanica)*.

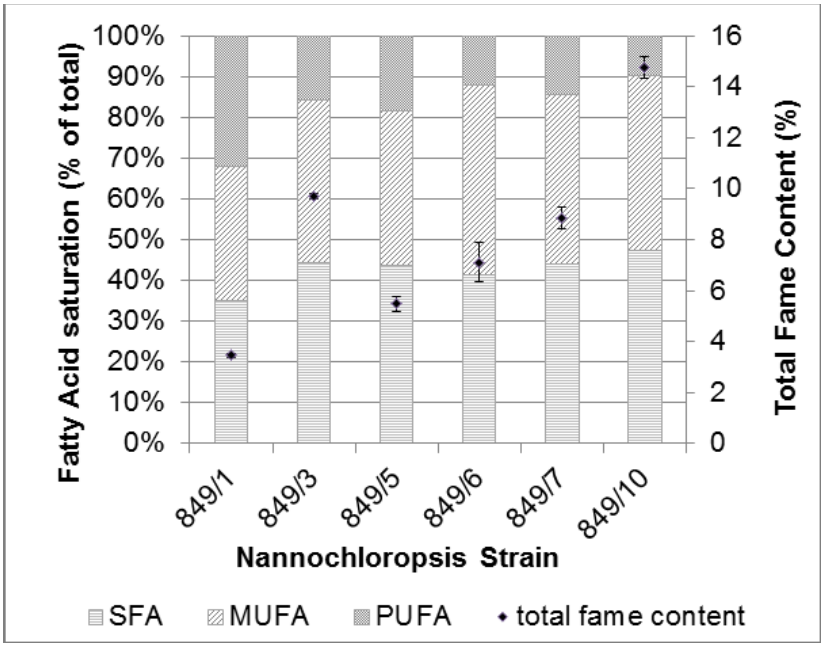
Figure 4. *Nannochloropsis salina* strain 849/3 grown under two salinity conditions. Panel A shows total FAME content change during culture growth and decline. exp: exponential, stat: stationary. Panel B Representative sample image of each condition during mid-exponential growth, with the scale bar indicating 1 µm.

435 **Figure 1**



436

437 **Figure 2**



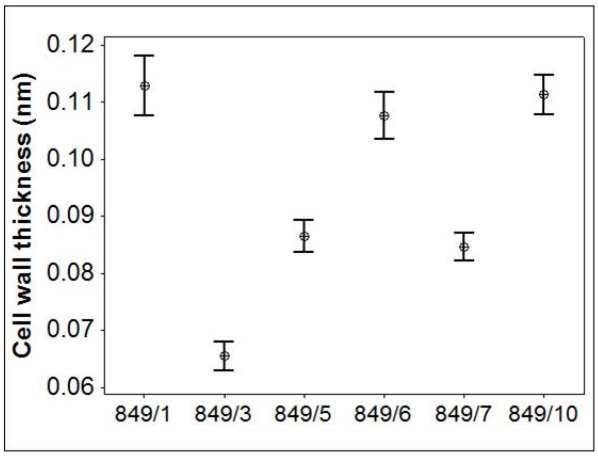
438

439 **Figure 3**

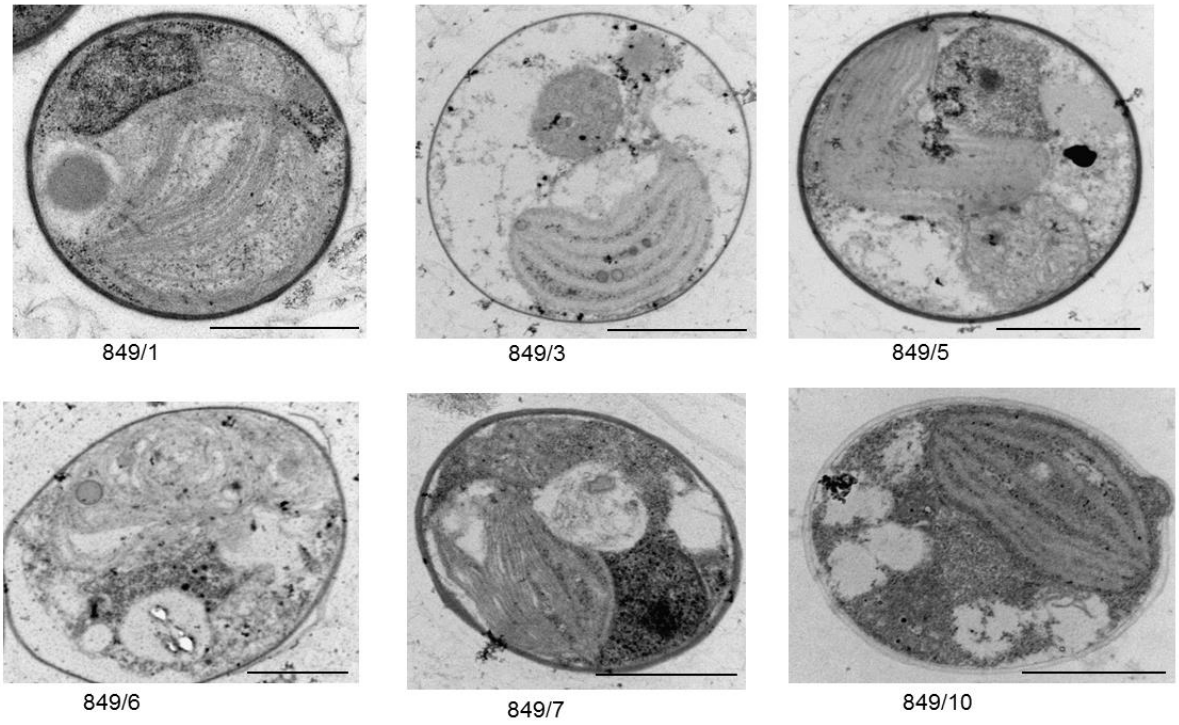
A

Strain	Average cell wall thickness (nm)	Average cell size (μm)
849/1	112.9 ± 5.9	2.36 ± 0.04
849/3	65.5 ± 2.8	2.50 ± 0.08
849/5	86.5 ± 3.2	2.77 ± 0.07
849/6	108.1 ± 4.7	2.31 ± 0.09
849/7	84.7 ± 2.8	2.59 ± 0.04
849/10	111.3 ± 3.9	2.47 ± 0.04

B

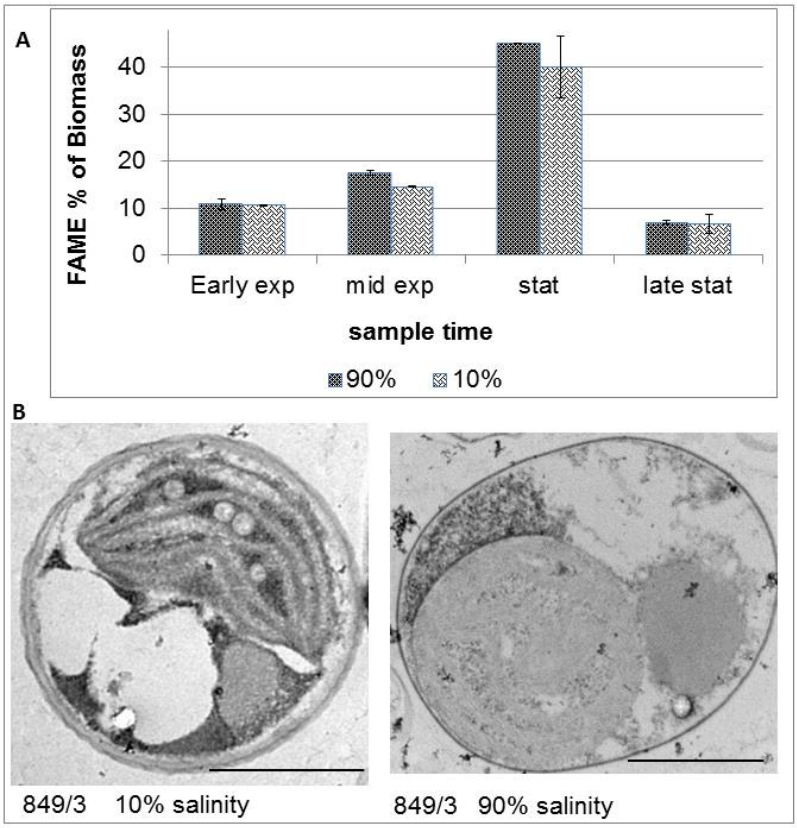


C



440

441 **Figure 4**



442

443