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Tailoring of the biochemical profiles of microalgae by employing mixotrophic cultivation

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Abstract

Mixotrophy, in marine microalgae, is the ability to uptake organic nutrients from the environment, in addition to, or as an alternative to, fixation of carbon through photosynthesis. Many microalgae are capable of mixotrophic and heterotrophic growth, which can be used to maximise culture density, while theoretically lowering production

costs compared to photoautotrophy. Here we show that, following screening of three microalgal species for mixotrophic and heterotrophic growth, *Tetraselmis suecica* and *Cyclotella cryptica* were capable of mixotrophic growth on glycerol, glucose and acetate and heterotrophically on glucose. *Phaeodactylum tricornutum* could only be cultured mixotrophically, but when cultured on glycerol cell density increased fourfold compared to photoautotrophic culture. Carbon allocation changed dependent upon carbon source and time of harvesting, with mixotrophic culture of *P. tricornutum* on glycerol resulting in lower cell length and increased total fatty acid content. These changes can be utilised in the development of a 'designer alga'.

Keywords

'designer algae', mixotrophy, aquaculture, biotechnology

1 Introduction

Microalgae are important organisms to a number of industries, such as: pharmaceuticals, nutracueticals, aquaculture feed and biofuels. Currently the costs associated with microalgal culture for commercial use are high, placed at €3.4 kg^{-1} for a theoretical production facility in Spain (Ruiz et al., 2016) and the culture of live feed account for around 30% of total hatchery operating costs for the production of mussel larvae (Coutteau and Sorgeloos, 1992). In this study mixotrophy is focussed upon as a potential mechanism to reduce the costs of microalgal culture. Mixotrophy has a number of advantages over photoautotrophic culture of microalgae, including a theoretical lower cost (Perez-Garcia et al., 2011), increased amount of biomass (Day and Tsavalos, 1996), in-

creased cell density (Kumar et al., 2018) and an increase in the target biochemical component (Pahl et al., 2010).

Microalgae are classically considered to be obligate photoautotrophs, obtaining their energy through photosynthetic fixation of carbon; however, there is evidence that many microalgae have the ability to assimilate organic carbon (Myers, 1962) or nitrogen from the environment (Droop, 1974). If the microalgae in question are able to assimilate the organic nutrients, and grow, in the dark they are termed heterotrophic, while if it can switch between heterotrophy and photoautotrophy, or can only utilise the organic carbon when it is also cultured in the presence of light, it is termed mixotrophic (Burkholder et al., 2008). Mechanisms of heterotrophy and mixotrophy vary between species and may include phagotrophy, the engulfing of other organisms, such as bacteria in the case of *Ochromonas danica* (Xiao and Ju, 2018), or osmotrophy, the uptake of dissolved organic carbon or nitrogen from the environment, displayed by *Chlorella vulgaris* (Spijkerman et al., 2017). The amount of carbon and nitrogen assimilated varies between species and culture conditions (Carpenter et al., 2018). Triggers to alter this assimilation may be linked to: particle density, irradiance, concentration of organic nutrients or pH amongst other factors (Thingstad et al., 1996). For example *C. cryptica* upregulates glucose uptake when incubated in the dark with glucose (Lylis and Trainor, 1973).

Mixotrophy requires the presence of appropriate uptake proteins, such as *Hup1* in *Chlorella kessleri* (Sauer and Tanner, 1989), and the ability to metabolise the uptaken carbon source (Droop, 1974). The time taken to up-regulate and express the proteins required for metabolism may lead to a lag phase in population growth such as the two day lag phase necessary for *Galdieria sulphuraria* (Gross and Schnarrenberger, 1995)

and a 24 hour lag phase for *Cyclotella cryptica* (Hellebust, 1971). The addition of an organic carbon source will also affect the carbon partitioning in the cell, changing the amount of carbon channelled through different metabolic pathways, dependant upon the requirements of the cell and the population growth stage (Brown, 1991). Acetate can be metabolised by binding with the acetyl-CoA protein and transported into the glycoxysome, directly incorporated in to the tricarboxylic acid cycle in the mitochondria, or incorporated directly in to fatty acid synthesis (Morales-Sánchez and Martinez-Rodriguez, 2015), this allows for an increase in respiration or an increase in carbohydrate accumulation as displayed by *Tetraselmis suecica* (Vitova et al., 2015). For *P. tricornutum* glycerol increases the synthesis of new triacylglyceride (TAG), leading to an increase in the C16:0 and C16:1 and Eicosapentaenoic acid (EPA) content as well as an increase in the synthesis of carbon storage carbohydrates (Villanova et al., 2017). Lipid and poly-unsaturated fatty acid accumulation is generally associated with stationary phase growth (Fidalgo et al., 1998). By utilising specific carbon sources and harvesting at specific stages of growth one can effectively tailor the biochemical profiles of the cells, developing a 'designer alga'. The application of this 'designer alga' to microalgal culture may be significant. All this may be achievable for a reduced cost through improved culture productivity and a tailored biochemical profile (Morales-Sánchez, 2013).

This study screens the ability of three species: *Tetraselmis suecica, Phaeodactylum tricornutum* and *Cyclotella cryptica* to grow mixotrophically or heterotrophically growth using: glucose, acetate or glycerol as a carbon source. The screening process was extended to three subcultures of each growth condition. Furthermore, the biochemical profile of the screened species were analysed, with the objective of utilising this information in the development of a 'designer alga' cultured in defined conditions and

harvested at a defined time.

2 Methodologies

2.1 Effects of repeated subculturing on growth of cultures

The three microalgal species screened for growth were bacteria-free strains of: *Phaeodactylum tricornutum* CCAP1055/1, *Tetraselmis suecica* CCAP 66/60 and *Cyclotella cryptica* CCMP332. These were obtained from the Culture Collection of Algae and Protozoa in Oban (*Phaeodactylum tricornutum* and *Tetraselmis suecica*) and the National Centre for Marine Algae and Microbiota in Bigelow (*Cyclotella cryptica*). These species were cultured photoautotrophically in 50 ml of f/2 medium, with additional silica for the two diatom species (Guillard and Ryther, 1962) and the population growth compared to cultures containing an additional 0.5 g l^{-1} yeast extract and a carbon source added at a concentration of 2 gl^{-1} carbon (5 g l^{-1} glucose, 5.12 g l^{-1} glycerol and 6.84 g l^{-1} acetate). All media had the pH adjusted to 8.0 using HCl or NaOH (Inolab pH probe 03060046, Germany). Triplicate cultures were incubated at 20 °C, with 100 rpm shaking for *T. suecica* and *C. cryptica*. For photoautotrophic and mixotrophic growth, cultures were maintained in a 12:12 light cycle at 40 microMol photons m^{-2} s^{-1} (QSL-100 1226, USA), while heterotrophic cultures were incubated in the dark by covering the culture flasks (Innova 44 shaking incubator, 900240247, USA). There was no additional gas supply. Gas exchange provided by the standard covered conical flask bung system.

These cultures were incubated for a 3 week period with two, 0.5 ml, samples aseptically removed every Monday, Wednesday and Friday to enumerate cell numbers using haemocytometry. After three weeks cultures were aseptically subcultured into fresh media (10 % *v/v* inocula) and this process repeated for a total of three subcultures. If cultures did not show an increase in cell numbers after the second subculture they were considered to be incapable of being cultured under the tested conditions and were not maintained for a third subculture. Following the third subculture, those strains which could be successfully cultured heterotrophically or mixotrophically were subcultured every 3-4 weeks. To compare the effects of heterotrophy and mixotrophy on the biochemistry of the algal species triplicate, 50 ml, cultures were harvested at the mid point of either growth or stationary phases. Of these samples, 1 ml was separated for cell counts and a further 1 ml for cell diameter determination. Subsequently the remaining culture was harvested (3721 g, 20 minutes, Heraeus Multifuge X3FR 41613936, Germany). The supernatant was discarded and the pellet resuspended in ultrapure Water before transfer to preweighted 50 ml falcon tubes. These were freeze-dried for a minimum of 24 hours, until dry (Christ Alpha 1-2 LD Plus, 12570, Germany) and weighted prior to biochemical analysis.

2.2 Biochemical analyses

2.2.1 Cell diameter measurement

Prior to utilising the one ml sample from the culture for cell diameter measurements, this was filtered through a 20 μm filter, then 0.1 ml was diluted with 10 ml of 0.9 % NaCl solution and the cell diameters measured using a Coulter counter (Beckman Coul-

ter, Multisizer 3 AK25124, UK). For all photoautotrophic cultures 0.5 ml was assayed due to low cell numbers present. The cell diameters were 'gated' as follows: *T. suecica* between 5-20 μm, *P. tricornutum* 2.603-15 μm and *C. cryptica* 5-20 μm. This was to ensure that any bacterial contamination from the processing of the sample did not affect the cell diameters measured.

2.2.2 Fatty acid analysis

Using a previously described direct derivitisation method (Slocombe et al., 2012), with minor modifications, the fatty acid profiles were analysed. To do this 10 mg of freeze-dried material was measured into 1.5 ml glass screw-capped vials, which had previously had 10 μl of internal standard (C23:0 at a concentration of 1 mg ml^{-1} in 2:1 chloroform-methanol) dried down using nitrogen gas (N-EVAP organomation 61220, Germany) in to the vial. To this vial 200 μl of hexane and 0.5 mL of 1 N Methanolic-HCl was added. These were flushed with nitrogen and heated at 85°C for two hours (Thermo-Heraeus oven 40756721, Germany). The samples were allowed to cool to room temperature and 0.25 mL of 0.9 % KCl was added with gentle mixing. The upper layer was removed and placed into a new glass vial. These were flushed with nitrogen and stored at -20 °C prior to analysis using gas chromatography (Shimadzu GC-2014 C1148440337, Japan).

2.3 Protein content

Protein content was measured spectrophotometrically in triplicate. Samples of 5 $\pm$0.25 mg of freeze-dried micro-algae were placed in screw-capped eppendorfs tubes and anal-

ysed using the Lowry assay (Lowry et al., 1951), as modified by Slocombe et al. (2013). This material was resuspended in 200 μl of 24 % TCA (*w/v*) and incubated at 95-100 °C for 15 minutes. Following this the samples were cooled and vortexed for 30 seconds to resuspend. Once cooled 600 μl of ultrapure water was added and the samples centrifuged (162,000 g, 20 minutes, Heraus picofuge 17). The resulting pellet was resuspended in 0.5 mL Lowry reagent (48:1:1 of 2 % Na_2CO_3 (anhydrous) in 0.1 N NaOH, 1 % NaK Tartrate (tetrahydrate) in ultrapure water and 0.5 % $CuSO_4$.5H2O in ultrapure water), vortexed and incubated at 55 °C for one hour. After allowing samples to cool they were centrifuged (162,000 g, 20 minutes, Heraus picofuge 17). Of the resulting supernatant 50 μl was transferred, in triplicate, to glass eppendorfs containing 1 ml of Lowry reagent and incubated for 10 minutes at room temperature. Subsequently 0.1 ml Folin-Phenol-water was added to each sample and vortexed immediately. They were incubated for 40 minutes and the absorbance measured at 550, 600, and 750 nm (glass cuvettes, Nicolet evolution 300 spectrometer EV3132101, UK). The protein content was calculated using a standard curve of known concentrations of bovine serum albumen.

2.4 Carbohydrate content

Changes in the carbohydrate content were measured using the Dubios carbohydrate assay (Dubois et al., 1956), modified for use in algal cultures (Slocombe et al., 2015). for analysis 5 $\pm$0.25 mg of freeze-dried micro-algae material was used from each triplicate culture. This sample was placed into screw-capped glass eppendorfs and resuspended in 0.5 ml of one molar sulphuric acid by vortexing. These were autoclaved to hydrolyse (121 °C for 15 minutes, Astell CA13N61102, UK). After autoclaving, samples were

placed on ice to cool before centrifugation (162,000 g, 10 minutes, Heraus picofuge 17 40802214, Germany). Triplicate samples of 10 μl of the hydrolysed material was transferred to glass test tubes. To this, 0.5 ml of 4 % phenol was added and gently vortexed. A further 2.5 mL of concentrated sulphuric acid was then added and again vortexed. Absorbances were measured (490 nm, quartz cuvettes, Nicolet evolution 300 spectrometer EV3132101, UK). The carbohydrate contents were calculated using a standard curve of glucose at known concentrations.

2.5 Chlorophyll and carotenoid content

Chlorophyll and carotenoid content was analysed spectrophotometrically using a modified ethanol extraction (Thrane et al., 2015). To do so, 3 ml of 96 % ethanol was added to 2 mg of the freeze-dried microalgae. This was then vortexed and left overnight at 4 °C. Of this sample 750 μl was extracted in triplicate and the absorbance of the sample measured between 400-700 nm at 1 nm intervals (POLARstar omega 415-2617, Germany). From these data the total chlorophyll and carotenoid content quantified using the scripts provided for use in R studio (v2) (Thrane et al., 2015).

2.6 Statistical analyses

The biochemical profiles of the microalgae tested were analysed for normality using the Kolmogorov-Smirnov test and if these data were normally distributed ($P>0.05$) subsequently tested for equal variances using Levene's test. If variances could be considered to be equal then one-way ANOVA and *post hoc* Tukey's test was utilised to understand

the differences in the relevant profile when comparing growth conditions. If samples were not normally distributed ($P<0.05$) then a Kruskall-Wallis and *post hoc* Dunn's non-parametric comparison were used to understand the changes in the component in question. These analyses were performed in Minitab (v18). When displayed graphically the mean cell number or %DW was presented $\pm$ the standard deviation. To analyse the fatty acid profiles of the organisms, data were initially placed in a dissimilarity matrix and the differences between groups analysed using ANOSIM. *Post hoc* the drivers of the differences between groups was understood using SIMPER, followed by graphical display using Non-metric Multidimensional Scaling (NMDS) in R studio (v2).

3 Results and discussion

It can be concluded that all of these screened strains are capable of mixotrophic growth under the conditions tested with glucose, acetate and glycerol as additional carbon sources (Fig: 1). In addition *T. suecica* and *C. cryptica* were capable of heterotrophic growth with glucose. For *T. suecica* and *P. tricornutum* the addition of this organic carbon source resulted in an increased cell density at stationary phase. The biochemical profile of the population changes with the addition of an organic carbon source, indicating a change in the carbon partitioning of the cells (Table: 1). The changes in the biochemical profiles of these organisms may be beneficial in the development of a 'designer alga'. The growth curves of the mixotrophic, or heterotrophic, populations were considered to be repeatable after three subcultures. Earlier subcultures were characterised by an extended lag phase and fewer cells, by volume, in the starting inocula. There was a reduction in the duration of the lag phase in all species and an increase in the number of cells inoculated after the second subculture. It was assumed that the

observed reduction in the lag phase may be due to the time required to upregulate the proteins necessary for uptake and metabolism of carbon (Radonjic et al., 2005). When cultured mixotrophically, the expression of these proteins is already upregulated, resulting in a reduction in the lag phase (Gross and Schnarrenberger, 1995). Therefore in future studies of mixotrophy, a mixotrophic starter culture should be employed.

3.1 *T. suecica*

Successful culture of *T. suecica* on all the carbon sources and heterotrophic culture with glucose is in agreement with previous studies, which demonstrated successful cultivation of *T. suecica* on glucose, heterotrophically (Day and Tsavalos, 1996) and mixotrophically (Cid et al., 1992) and mixotrophically on acetate (Azma et al., 2011). There was an increased cellular density in the mixotrophic and heterotrophic cultures, both across the whole population growth (Fig: 1A) and in terms of dry weight at harvesting (Table: 1). Further improvements through culture optimisation may produce dry weights of 3 g l^{-1} such as those observed in closely related species (Day and Tsavalos (1996); Cid et al. (1992)). Following culture optimisation heterotrophically cultured *Tetraselmis* spp. populations attained cell numbers of 500-700 x10^4ml^{-1} which is approximately double that found by this species without optimisation (Table: 1). To achieve such increased cell density 40 g l^{-1} glucose was required, suggesting a direction for future culture optimisation, alongside optimising the biochemical profile (Morales-Sánchez and Martinez-Rodriguez, 2015).

For *T. suecica* the: cell diameters, carbohydrate and protein contents were normally

distributed (Kolmogorov-Smirnov $P>0.05$) and variances can be considered to be equal (Levene's test $P>0.05$, $F=1.40, 0.59, 0.82$ respectively). Total fatty acid (TFA), chlorophyll and carotenoid contents were not normally distributed (Kolmogorov- Smirnov $P<0.05$).

Cell diameters varied with harvesting time and culture conditions (ANOVA and *post hoc* Tukey's test, $F=9.87$, $P<0.05$, df= 10). There was a significantly greater mean cell diameter in photoautotrophic cultures at stationary phase (Fig: 2A), compared to mixotrophic glycerol cultures harvested at growth stage and heterotrophic glucose cultures also harvested at growth phase (mean= 8.67, s.d= 0.46 μm compared to mean= 10.87, s.d= 0.13 μm and mean= 10.33, s.d= 0.36 μm respectively). There was a significant difference in the chlorophyll contents dependant upon method of culture (Kruskal-Wallis and *post hoc* Dunn's non-parametric comparison, $H=17.09$, df= 9, $P<0.05$). There was a significantly reduced chlorophyll content when *T. suecica* is cultured heterotrophically (Fig: 2B) and harvested during stationary phase, compared to the same culture conditions, when harvested during growth phase (median= 0.21, range= 0.28 %DW compared to median= 3.09, range= 4.46 %DW). There was no significant difference in the carotenoid contents (Kruskal-Wallis, $H=15.98$, df= 9, $P>0.05$).

The accumulation of carbohydrates was affected by method of culture (ANOVA and *post hoc* Tukey's test, $P<0.05$, $F=16.1$, df=9, Fig: 2C). Photoautotrophically cultured samples contained a significantly lower carbohydrate content compared to heterotrophically cultured samples, irrespective of harvesting time (mean= 2.82, s.d= 0.7 %DW and mean= 43.78 %DW, s.d= 5.02 harvested at stationary phase respectively). Furthermore, mixotrophic cultures containing acetate harvested at stationary phase and mixotrophic

cultures containing glucose also had a significantly greater carbohydrate content compared to photoautotrophic cultures. *T. suecica* strains isolated from north-west Spain also displayed an increase in carbohydrate content, per cell, when cultured mixotrophically with glucose (Cid et al., 1992) as did the *Tetraselmis* spp. KY114885 (Lari et al., 2019). This accumulation of carbohydrates per lipids has been observed in both heterotrophy (Day and Tsavalos, 1996) and mixotrophy with glucose (Lari et al., 2019). Mixotrophy and heterotrophy may have a similar effect upon the cell cycle as nitrogen limitation, leading to an increase in the accumulation of carbohydrates or lipids as energy reserves (Villanova et al., 2017). The response to accumulate carbohydrates or lipids appears to be species and strain specific as the glucose metabolism pathway has only been putatively identified (Morales-Sánchez and Martinez-Rodriguez, 2015) and the carbon source accumulated form a pool of C3 precursors which is then partitioned dependant upon the needs of the cell (Vitova et al., 2015), either through synthesis of TAG (Villanova et al., 2017), formation of carbohydrates or direct metabolism in the TCA cycle (Morales-Sánchez and Martinez-Rodriguez, 2015). The causes and drivers for these changes in carbon partitioning during mixotrophic culture is currently poorly understood (Vitova et al., 2015).

The protein contents of the populations varied significantly (ANOVA and *post hoc* Tukey's test, $P<0.05$, $F=6.28$, $df=9$). *T. suecica* cultured photoautotrophically during growth or stationary phase had a significantly lower protein content compared to cultures grown with acetate, harvested at growth phase (mean= 5.42, s.d=1.23 %DW and mean=16.11, s.d=4.78 %DW for stationary phase cultures respectively). This contrasts with other strains of *T. suecica*, where mixotrophic culture with glucose also resulted in an increased protein content per cell (Cid et al., 1992).

There was a significant difference between the TFA contents of the different conditions (Kruskal-Wallis and *post hoc* Dunn's non-parametric comparison, $P<0.05$, H=23.8, df=9). *T. suecica* cultured mixotrophically on glucose and harvested during growth phase and heterotrophically on glucose and harvested during stationary phase both had significantly lower TFA content compared to cultures grown in the presence of acetate and harvested during stationary phase (mixotrophic culture with acetate median TFA=10.53, range=17.63 %DW, mixotrophic culture with glucose harvested during stationary phase median TFA=1.24 range=1.05 %DW). This is in agreement with other heterotrophically cultured *Tetraselmis* spp. which were characterised by a reduction in the TFA and unsaturated fatty acid contents (Day and Tsavalos, 1996). This response appears to be strain and species specific, as *Tetraselmis* spp. KY114885 shows an increased lipid accumulation with the same amount of glucose in the medium (Lari et al., 2019). The commensurate reduction in the chlorophyll content of the population may indicate a reduction in the number of plastids in the cell and so a reduction in lipid synthesis during heterotrophy. The fatty acid profile was displayed graphically by NMDS and there was a significant difference between the culture conditions (ANOSIM, $P<0.05$, R=0.66, Fig: 2D). Photoautotrophic culture was associated with a significantly increased content of C18:0 when harvested during growth phase (SIMPER, $P<0.05$), compared to all other culture conditions, and an increase in C18:3 n-3, 18:2 n-6, EPA and 20:1 n-9 content compared to mixotrophic culture with glucose. When harvested at stationary phase, photoautotrophic cultures were not significantly different in fatty acid profile compared to any other conditions, except mixotrophic culture with glucose. Under these conditions photoautotrophic cultures had a significantly greater content of C16:0, 17:0, 18:1 n-9, 18:2 n-6 and 18:3 n-3. This shift away from saturated

and polyunsaturated fatty acids, in favour of monounsaturated fatty acids was observed in *Tetraselmis* spp. KY114885 when cultured mixotrophically with glucose (Cid et al., 1992) and both mixotrophy and heterotrophy have been shown to shift fatty acid profiles in favour of mono or saturated fatty acids, including *Chlorella vulgaris* (Katiyar et al., 2018), *Ettalia*. spp (Kam et al., 2017) and *Tetraselmis*. spp (Day and Tsavalos, 1996). This accumulation of shorter chain and saturated fatty acids may be a change in the carbon partitioning with the shift to neosynthesis of lipids due to the increased availability of C3 precursors which primarily comprise of shorter chain fatty acids (Villanova et al., 2017).

3.2 *P. tricornutum*

It was only possible to cultivate *P. tricornutum* mixotrophically, with the culture crashing rapidly when incubated in the dark. This strain's inability to be cultured heterotrophically is in agreement with previous studies on other strains of this species (Garcí et al., 2000), though it is possible, through genetic modification, to develop a strain that is capable of being cultured heterotrophically (Zaslavskaia et al., 2001). In all cases mixotrophy resulted in an increased cell density, in this instance approximately four times that of photoautotrophic culture (Table: 1) and cultures reached stationary phase more quickly (Fig: 1B). This could be further improved through culture optimisation, for example cultures containing ammonia chloride and glycerol have been shown to give a tenfold improvement in cell density compared to photoautotrophic culture (Garcí et al., 2000). By reducing the content of glycerol by approximately 0.5 g l^{-1} this led to a commensurate reduction in the cell numbers to 2 x10^4ml^{-1}, suggesting that the supply of glycerol

is an important factor to be considered in culture optimisation (Villanova et al., 2017) and the mechanisms of uptake and metabolism of glycerol are important areas for future study.

The cell lengths, carbohydrate and protein contents were normally distributed (Kolmogorov-Smirnov $P>0.05$) and variances can be considered to be equal (Levene's test $P>0.05$, f=1.15, 0.42 and 1.14 respectively). TFA, chlorophyll and carotenoid contents could not be considered to be normally distributed (Kolmogorv- Smirnov $P<0.05$).

Cell lengths of *P. tricornutum* varied when cultured in the different conditions (ANOVA and *post hoc* Tukey's test, $P<0.05$, F=52.52, df=7, Fig: 3A). There was a significantly greater mean cell lengths in photoautotrophic and glucose cultures at growth phase, compared to all other cultures (mean=4.90, s.d=0.05 μm for photoautotrophic cultures in growth phase). Furthermore, for photoautotrophic cultures harvested at stationary phase, there was no significant difference between these and glycerol cultures harvested at growth stage and glucose cultures harvested at stationary phase (mean= 4.04, s.d= 0.31 μm for photoautotrophic cultures harvested at stationary phase). These were significantly shorter in length compared to photoautotrophic cultures harvested during growth phase, however they were also significantly longer than glycerol cultures harvested during stationary phase or *P. tricornutum* cultured with acetate, irrespective of harvesting time. For all other culture treatments cell lengths were significantly greater when harvested during growth phase compared to stationary phase, except for cultures containing acetate (Fig: 3A).

Chlorophyll contents varied depending on time and culture conditions (Kruskal-

Wallis and *post hoc* Dunn's non-parametric comparison, $P<0.05$, $H=17.95$, $df= 7$, Fig: 3B). Culture with acetate and harvest during stationary phase resulted in a significantly reduced chlorophyll content, compared to culture with glycerol or photoautotrophic cultures when harvested during stationary phase (median=4.50, range= 0.70 %DW compared to median= 0.37, range= 0.21 %DW for acetate and photoautotrophic culture respectively). This variation was also present in the carotenoid contents of the populations (Kruskal-Wallis and *post hoc* Dunn's non-parametric comparison, $P>0.05$, $H=19.73$, $df= 7$). Cultures with acetate harvested at stationary and growth phase had a significantly increased carotenoid content compared to photoautotrophic cultures (median= 3.02, range=0.52 %DW compared to median= 0.20, range= 0.20 %DW for acetate and photoautotrophic cultures harvested at stationary phase respectively) while mixotrophic culture with glycerol harvested at stationary phase had a significantly lower carotenoid content. The changing chlorophyll and carotenoid contents may be due to a changed need for photosynthesis to provide ATP for uptake of the organic carbon in the media, with acetate requiring ATP from cyclic photophosphorylation while glycerol uptake has a lower requirement for photosynthetically derived ATP (Droop, 1974).

Carbohydrate contents also varied significantly (ANOVA and *post hoc* Tukey's test, $P<0.05$, $F=11.36$, $df=7$, Fig: 3C). Culture with glucose or glycerol harvested during stationary phase had a significantly greater carbohydrate content when compared to photoautotrophic cultures harvested at any time (mean=19.91, s.d=5.91% DW compared to mean=3.77, s.d=1.38 %DW for glucose and photoautotrophy harvested at stationary phase respectively). This accumulation of carbohydrates during stationary phase may indicate that the glycerol excess glycerol drives the cells to accumulate energy storage components in agreement with other strains (Villanova et al., 2017) and species (Lari

et al., 2019). There was no significant difference between the protein contents of any cell cultured under any other conditions tested (ANOVA, $P>0.05$, df=7, F= 2.05).

There is a significant difference between the TFA of samples (Kruskal-Wallis and *post hoc* Dunn's non-parametric comparison, $P<0.05$, H=16.33, df=7). *P. tricornutum* cultured mixotrophically on glycerol to stationary phase had a significantly higher TFA content compared to all glucose or photoautotrophic cultures harvested during growth phase (median=15.18, range= 6.63 %DW compared to median=2.92, range= 5.03 %DW for glycerol compared to photoautotrophy harvested during stationary phase respectively). The variations in fatty acid compositions were described using NMDS (Fig: 3D). There was a significant difference in the groupings (ANOSIM, $P<0.05$, R=0.42)and there was a significant difference in the fatty acid contents (SIMPER, $P<0.05$). Samples cultured with glucose all had an increase in C22:0 and C24:0 compared to photoautotrophic culture, while glycerol containing cultures harvested at stationary phase were associated with an increase in 17:1 during growth phase and C14:0, C18:1 n-9 and C18:3 n-6 during stationary phase, irrespective of the photoautotrophic culture sampling time. Increased accumulation in fatty acids during stationary phase has previously been observed in mixotrophically cultured *P. tricornutum* strain CCAP 1055/3. It has been suggested that glycerol has a similar effect upon the cell as nitrogen limitation, leading to an increased synthesis of both TAG, via the Kennedy pathway, and unsaturated fatty acids through conversion of existing polar glycolipids (Villanova et al., 2017).

3.3 *Cyclotella cryptica*

C. cryptica was successfully cultivated mixotrophically on all three substrates tested and heterotrophically on glucose (Fig: 1C). The inability of *C. cryptica* to be cultured using acetate or glycerol heterotrophically has previously been noted, with centric diatoms being considered to be incapable of heterotrophy on these substrates (Hellebust and Lewin, 1977). The ability to be cultured heterotrophically on glucose has been shown to be linked to a dark inducible glucose uptake protein (Lylis and Trainor, 1973), tentatively identified in the genome (Traller et al., 2016). When cultured mixotrophically or heterotrophically *C. cryptica* cell numbers did not exceed photoautotrophy (Table: 1) and the doubling time is much lower than the 14 hours predicted as the theoretical maximum (Hellebust, 1971) which suggests that a factor other than organic carbon concentration may be limiting. The population size at stationary phase was lower than the a *Cyclotella* spp. photoautotrophically cultured in a silica optimised culture (Li et al., 2017). As *C. cryptica* is known to have a greater requirement for silica, this suggest that this particular media competent should be optimised (Traller et al., 2016), alongside improving the understanding of mechanisms for uptake and metabolism of both glycerol and acetate in the genome.

The cell diameters were normally distributed (Kolmogorov-Smirnov $P>0.05$) and variances can be considered to be equal (Levene's test, $P>0.05$, $f=0.45$). Neither carbohydrate, protein, TFA, chlorophyll nor carotenoid content could not be considered to be normally distributed (Kolmogorov-Smirnov, $P<0.05$).

The cell diameters of *C. cryptica* cultured in the different conditions varied signif-

icantly (ANOVA and *post hoc* Tukey's test, P<0.05, F=9.00, df= 7, Fig: 4A). The cell diameter was significantly reduced when mixotrophically cultured with acetate and harvested during growth phase. Chlorophyll contents were significantly different (Kruskal-Wallis and *post hoc* Dunn's non-parametric comparison, P<0.05, df=9, H=23.7). Photoautotrophic cultures, when harvested a stationary phase, had a significantly greater chlorophyll content compared to mixotrophic glucose cultures harvested at growth phase and heterotrophic cultures harvested at stationary phase (median=1.63, range= 0.40 %DW compared to median= 0.060, range= 0.019 %DW respectively). A reduction was also observed in carotenoid contents (Kruskal-Wallis and *post hoc* Dunn's non-parametric comparison, P< 0.05, H=21.71, df=9) with photoautotrophic cultures containing an increased carotenoid content compared to mixotrophic cultures with glucose, harvested at stationary phase (Fig: 4B).

Photoautotrophic populations, irrespective of harvest time, had a significantly reduced carbohydrate content compared to heterotrophic cultures harvested at any time (Kruskal-Wallis and *post hoc* Dunn's non-parametric comparison, P<0.05, F=27.06, df=9, Fig: 4C). An accumulation of carbohydrates during heterotrophic culture has also been observed in the *C. cryptica* strain UTEX 1269 when cultured heterotrophically with glucose (Pahl et al., 2010). There was a significant difference in the protein contents dependant upon time of harvest and culture conditions (Kruskal-Wallis and Dunn's non-parametric comparison, P<0.05, F=22.27, df=9). *C. cryptica* cultured photoautotrophically and harvested during stationary phase had a significantly lower protein content compared to mixotrophically with glycerol, irrespective of harvesting stage (median=1.51, range= 0.12% DW compared to median= 7.35, range= 2.25, for stationary phase harvesting respectively).

As a bulk component TFA also varied significantly (Kruskal-Wallis and *post hoc* Dunn's non-parametric comparison, $P<0.05$, $F=17.37$, $df=9$), with mixotrophic cultures grown with glycerol harvested during growth stage containing a higher TFA content compared to mixotrophic cultures grown with glucose, irrespective of harvesting time (median=4.85, range=6.07 %DW, compared to 0.20, range= 2.51 respectively). The variations in fatty acid compositions were displayed graphically using NMDS (Fig: 4D). There was a significant difference in the groupings (ANOSIM, $R=0.4035$, $P<0.05$). In agreement with the reduction in the total fatty acid content during heterotrophic culture, there was also a signification reduction the contents of C17:0 and EPA (SIMPER, $P<0.05$). This reduction in TFA with heterotrophy, coupled with the lower chlorophyll and carotenoid contents suggests a reduction in the number of plastids and so fatty acid synthesis. This reduction is also observed in this study in *T. suecica* and is not uncommon in heterotrophically cultured microalgae (Morales-Sánchez and Martinez-Rodriguez, 2015), although is in contrast to the *C. cryptica* strain UTEX 1269 which displayed no such reduction in fatty acid accumulation (Pahl et al., 2010).

3.4 Potential applications and future directions

By increasing the cellular density of the microalga when cultured heterotrophically or mixotrophically the potential applications of these 'designer algae' are broad. As an aquaculture feed an increased cellular density and a biochemical profile high in fatty acids such as EPA and docosahexaenoic acid (DHA) may be a method of maximising growth and survival, for a reduced cost (Leonardos and Lucas, 2000) with enhanced

polyunsaturated fatty acid content linked to increased survival of *M. edulis* larvae (Helm and Laing (1987); Martínez-Fernández et al. (2006)). During early larval stages, smaller cells could be used in the feed, maximising the number of larvae which survive and develop further (Brown, 1991). *T. suecica* cultured with additional carbon resulted in an increased accumulation of carbohydrate (%DW) which may be important in bioethanol production (Bondioli et al., 2012). This study utilises a small scale cultivation method thus understanding of the potential viability of any of these substrates and conditions at larger scale is beyond the scope of this project.

4 Conclusions

T. suecica and *C. cryptica* could be cultured, mixotrophically, with the three organic carbon sources screened and heterotrophically with glucose, while *P. tricornutum* was capable of mixotrophic growth, but at four times the cell density of photoautotrophy. Carbon partitioning changed depending upon harvesting time and carbon source; for example, mixotrophic culture on glycerol resulted in increased protein for *C. cryptica*, while *P. tricornutum* had shorter cell lengths and increased TFA. These changes reflect species specific responses to altered availability of carbon. Utilising these changes in carbon partitioning enables the tailoring of microalgal biochemical profiles to develop 'designer algae'.

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croalgae used in this study.

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Figure captions

Fig: 1. The mean cell numbers of the three species screened, A: *T. suecica*, B: *P. tricornutum* and C: *C. cryptica*. The key indicates the carbon source and method of cultivation. All cell numbers are $\pm$ standard deviation.

Fig: 2. The biochemical profile of *T. suecica* harvested at growth and stationary phase $\pm$ standard deviation. A: mean cell diameter (μm), B: mean chlorophyll and carotenoid contents (%DW) and C: mean protein, carbohydrate and TFA content (%DW). * indicates that the component is significantly greater than photoautotrophic culture harvested at stationary phase. D: ordination of fatty acid compositions utilising NMDS. The key indicates the method of cultivation, carbon source and time of harvesting.

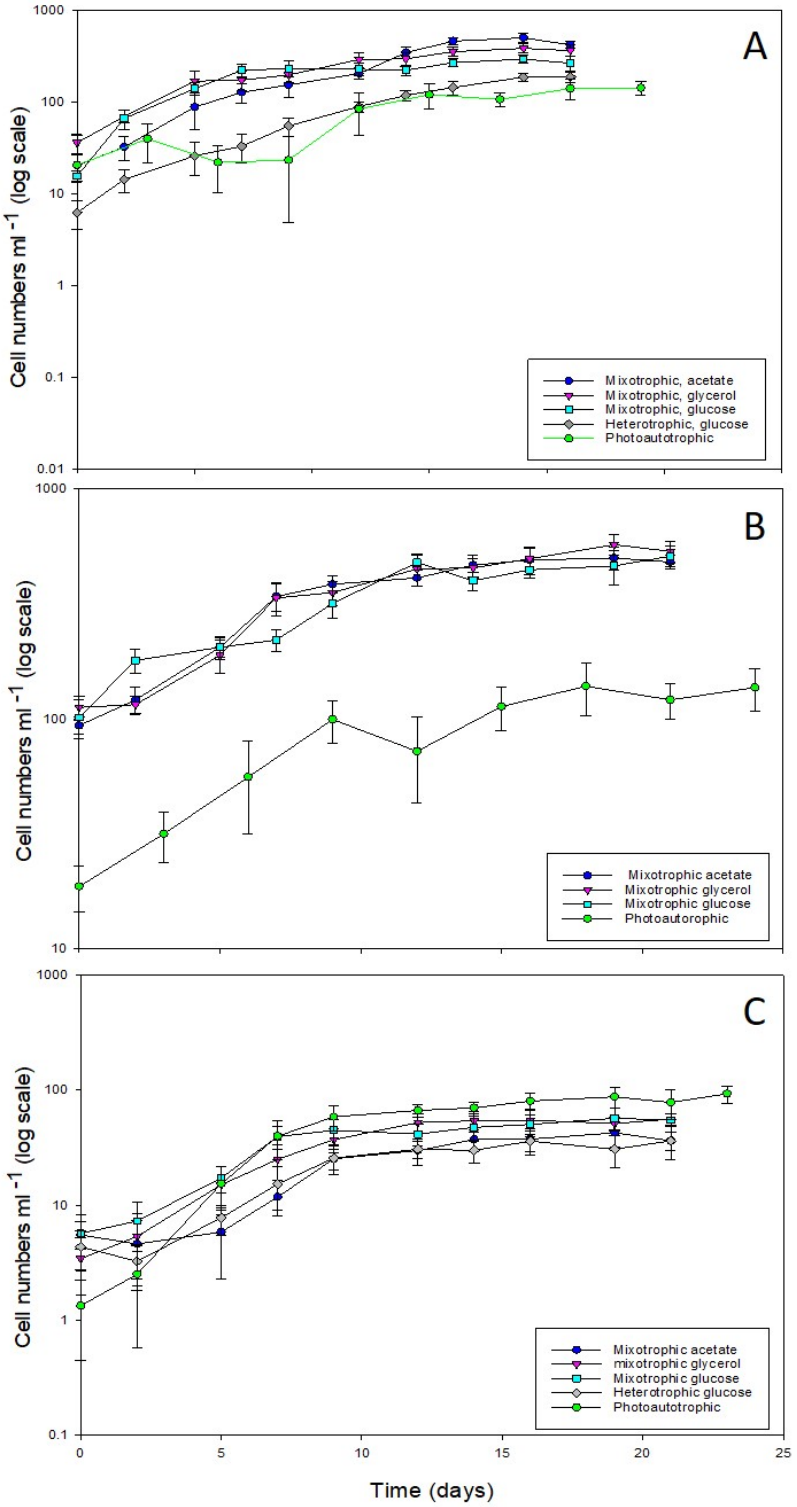
Fig: 3. The biochemical profile of *P. tricornutum* harvested at growth and stationary phase $\pm$ standard deviation. A: mean cell diameter (μm), B: mean chlorophyll

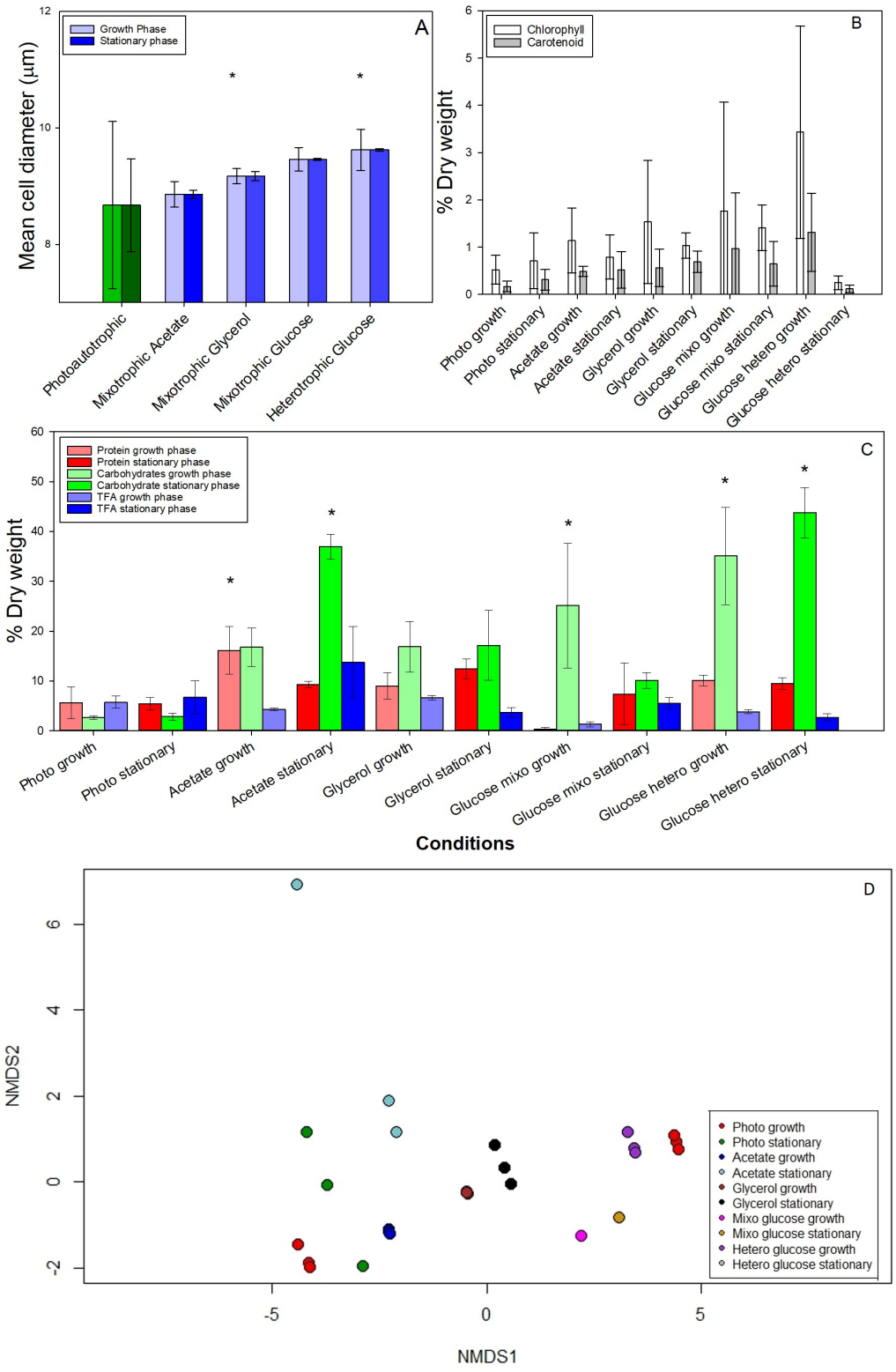
and carotenoid contents (%DW) and C: mean protein, carbohydrate and TFA content (%DW). * indicates that the component is significantly greater than photoautotrophic culture harvested at stationary phase and - that it is significantly lower. D: ordination of fatty acid compositions utilising NMDS. The key indicates the method of cultivation, carbon source and time of harvesting.

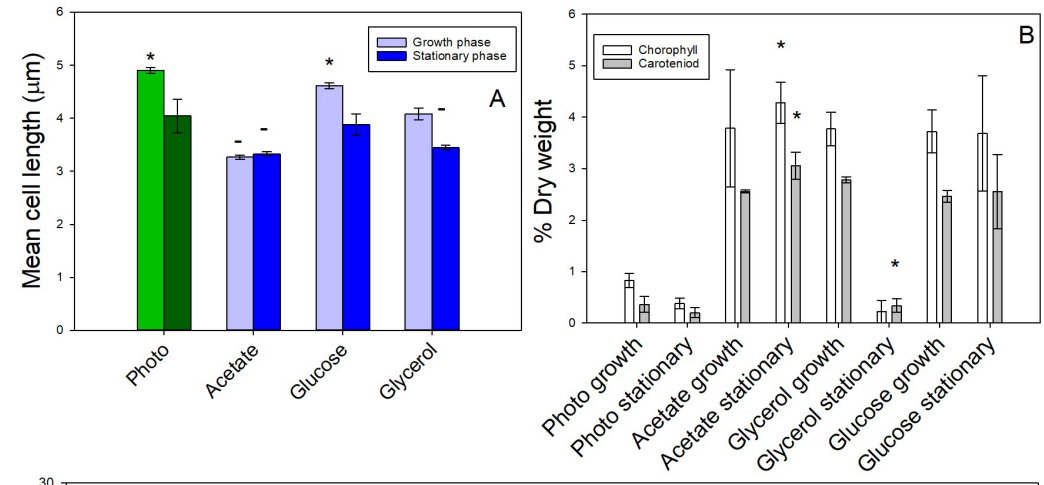
Fig: 4. The biochemical profile of *C. cryptica* harvested at growth and stationary phase $\pm$ standard deviation. A: mean cell diameter (μm), B: mean chlorophyll and carotenoid contents (%DW) and C: mean protein, carbohydrate and TFA content (%DW). * indicates that the component is significantly greater than photoautotrophic culture harvested at stationary phase and - that it is significantly lower. D: ordination of fatty acid compositions utilising NMDS. The key indicates the method of cultivation, carbon source and time of harvesting.

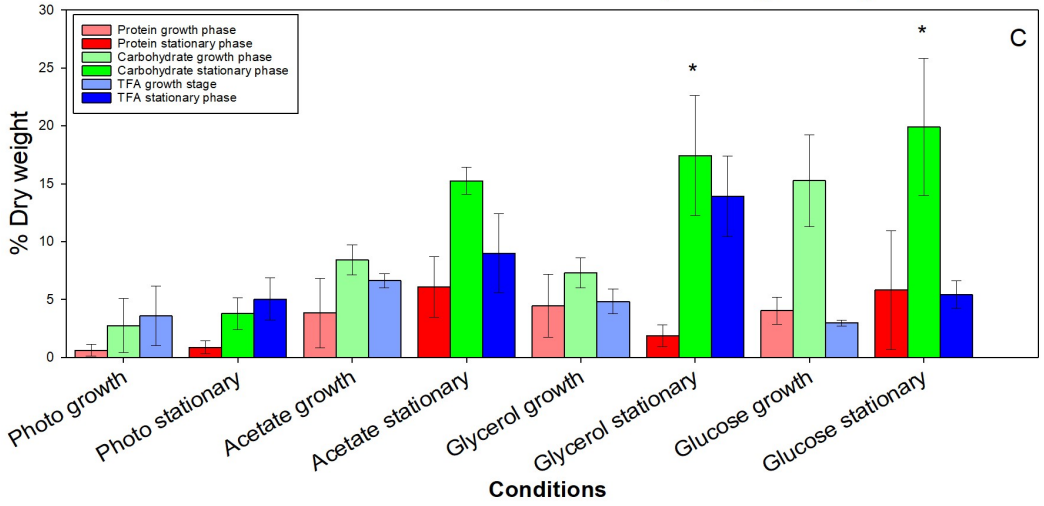
Table: 1. Summary of the changes in the biochemical profiles of the three species, for the named conditions and harvesting times. + indicates that the biochemical profile in question is statistically significant greater than photoautotrophy harvested at stationary phase, - that it is significantly lower and = that there is no significant difference.

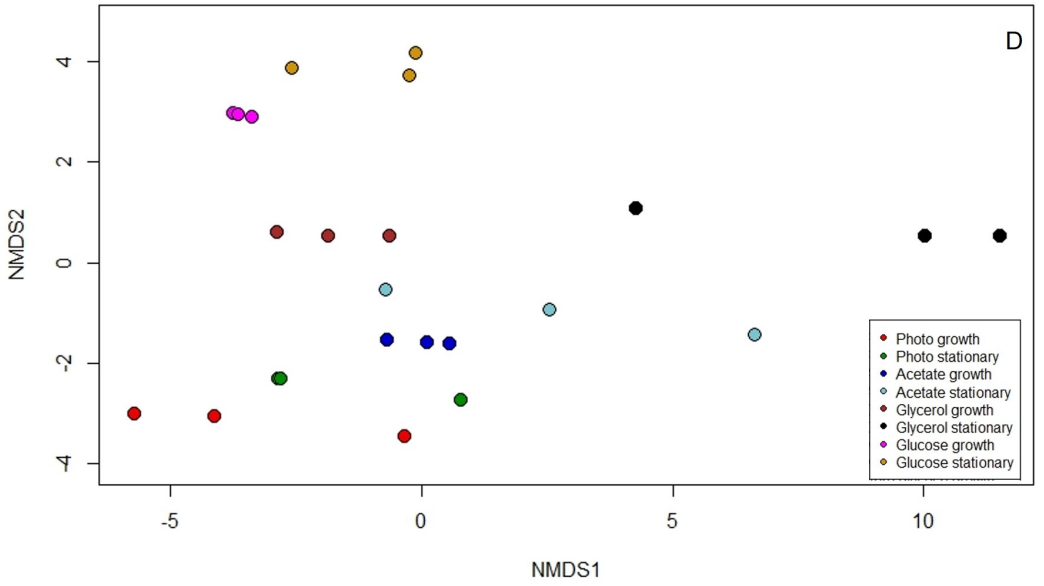
5 Tables and Figures

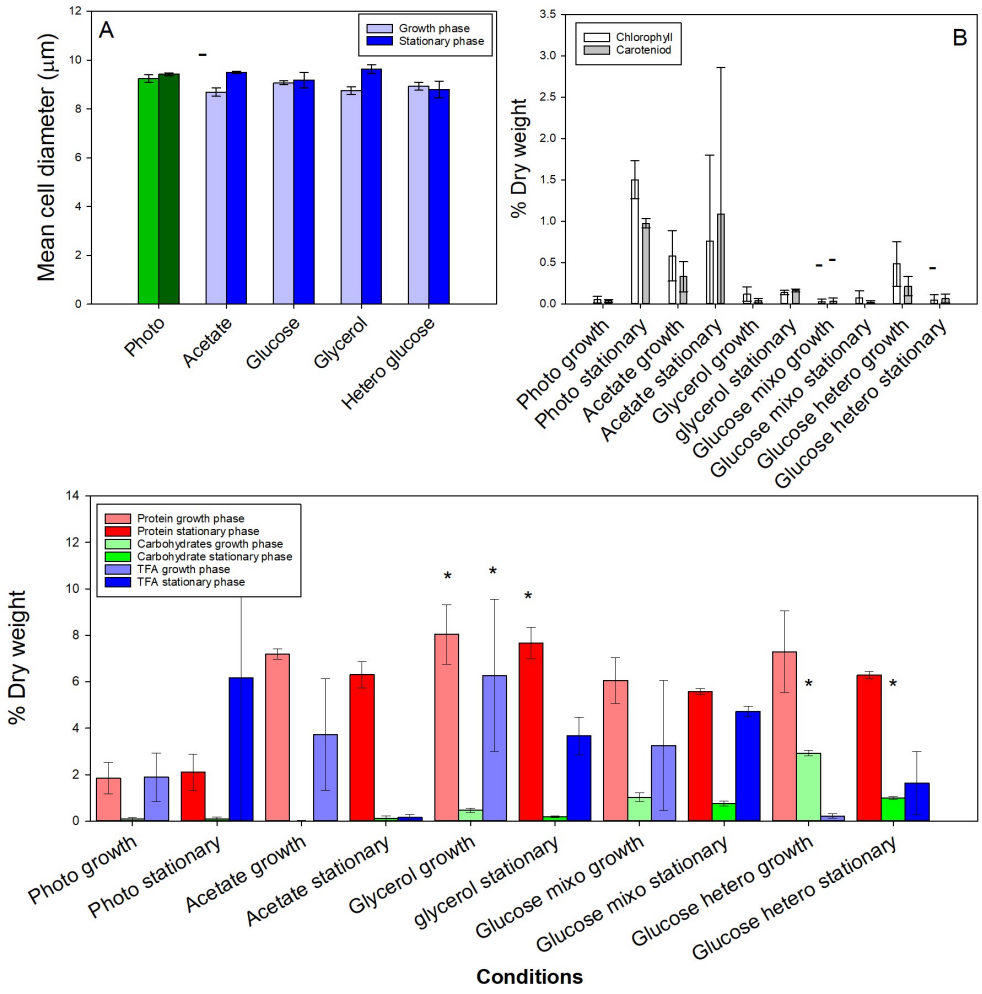



A
Growth Phase
Stationary phase
Mean cell diameter (µm)
Photoautotrophic
Mixotrophic Acetate
Mixotrophic Glycerol
Mixotrophic Glucose
Heterotrophic Glucose
B
Chlorophyll
Carotenoid
% Dry weight
Photo growth
Photo stationary
Acetate growth
Acetate stationary
Glycerol growth
Glycerol stationary
Glucose mixo growth
Glucose mixo stationary
Glucose hetero growth
Glucose hetero stationary
C
Protein growth phase
Protein stationary phase
Carbohydrates growth phase
Carbohydrate stationary phase
TFA growth phase
TFA stationary phase
% Dry weight
Conditions
D
NMDS2
NMDS1
Photo growth
Photo stationary
Acetate growth
Acetate stationary
Glycerol growth
Glycerol stationary
Mixo glucose growth
Mixo glucose stationary
Hetero glucose growth
Hetero glucose stationary


A
Mean cell length (μm)
Growth phase
Stationary phase
Photo
Acetate
Glucose
Glycerol
B
% Dry weight
Chorophyll
Caroteniod
Photo growth
Photo stationary
Acetate growth
Acetate stationary
Glycerol growth
Glycerol stationary
Glucose growth
Glucose stationary


C
% Dry weight
Protein growth phase
Protein stationary phase
Carbohydrate growth phase
Carbohydrate stationary phase
TFA growth stage
TFA stationary phase
Photo growth
Photo stationary
Acetate growth
Acetate stationary
Glycerol growth
Glycerol stationary
Glucose growth
Glucose stationary
Conditions


D
NMDS2
NMDS1
Photo growth
Photo stationary
Acetate growth
Acetate stationary
Glycerol growth
Glycerol stationary
Glucose growth
Glucose stationary


A
Growth phase
Stationary phase
Mean cell diameter (µm)
Photo
Acetate
Glucose
Glycerol
Hetero glucose
B
Chlorophyll
Caroteniod
% Dry weight
Photo growth
Photo stationary
Acetate growth
Acetate stationary
Glycerol growth
glycerol stationary
Glucose mixo growth
Glucose mixo stationary
Glucose hetero growth
Glucose hetero stationary
Protein growth phase
Protein stationary phase
Carbohydrates growth phase
Carbohydrate stationary phase
TFA growth phase
TFA stationary phase
% Dry weight
Conditions
D
Photo growth
Photo stationary
Acetate growth
Acetate stationary
Glycerol growth
Glycerol stationary
Mixo glucose growth
Mixo glucose stationary
Hetero glucose growth
Hetero glucose stationary
NMDS2
NMDS1

Conditions	Population growth phase	Mean cell counts (x10^4 ml^{-1})	Mean dry weight (g)	cell diameter (μm)	Protein content (%DW)	Carbohydrate content (%DW)	Total fatty acid content (%DW)	Chlorophyll content (%DW)	Caroteniod content (%DW)
T. suecica									
Photoautotrophic	Growth	18	0.32	=	=	=	=	=	=
	Stationary	66	1.29	=	=	=	=	=	=
Acetate	Growth	161	0.71	=	+	=	=	=	=
	Stationary	326	1.45	=	=	+	=	=	=
Glycerol	Growth	169	0.46	+	=	=	=	=	=
	Stationary	282	1.13	=	=	=	=	=	=
Mixotrophic Glucose	Growth	22	0.27	=	=	+	=	=	=
	Stationary	351	1.66	=	=	=	=	=	=
Heterotrophic Glucose	Growth	68	0.79	+	=	+	=	=	=
	Stationary	250	1.06	=	=	+	=	=	=
P. tricornutum									
Photoautotrophic	Growth	126	0.49	+	=	=	=	=	=
	Stationary	164	1.20	=	=	=	=	=	=
Acetate	Growth	378	0.43	-	=	=	=	=	=
	Stationary	434	0.68	-	=	=	=	+	+
Glycerol	Growth	340	0.76	=	=	=	=	=	=
	Stationary	434	1.98	-	+	=	=	=	+
Glucose	Growth	183	0.28	+	=	=	=	=	=
	Stationary	378	0.39	=	+	=	=	=	=
C. cryptica									
Photoautotrophic	Growth	33	0.23	=	=	=	=	=	=
	Stationary	60	1.49	=	=	=	=	=	=
Acetate	Growth	28	0.32	-	=	=	=	=	=
	Stationary	58	1.74	=	=	=	=	=	=
Glycerol	Growth	38	1.62	=	=	+	+	=	=
	Stationary	61	1.86	=	=	+	=	=	=
Mixotrophic Glucose	Growth	6	2.02	=	=	=	=	-	-
	Stationary	12	2.35	=	=	=	=	=	=
Heterotrophic Glucose	Growth	30	0.32	=	+	=	=	=	=
	Stationary	35	1.62	=	+	=	=	-	-