# Use of crude glycerol for mixotrophic culture of *Phaeodactylum tricornutum*

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## Abstract

Crude glycerol is a waste stream from biodiesel production, which has previously been shown to be useable for mixotrophic cultivation of microalgae; however, at high concentrations the presence of contaminants may limit culture growth. In this study two previously identified methods of contaminant removal from crude glycerol were trialled for use in mixotrophic culture of *Phaeodactylum tricornutum*. Neither crude glycerol without contaminant removal, or crude glycerol which has had contaminants removed through calcium precipitation, lead to culture growth of *P. tricornutum*. However, pH adjusted crude glycerol gave comparable cell densities ($6.03 \pm 0.24$ x$10^6$ cells ml$^{-1}$ compared to $5.66 \pm 0.15$ x$10^6$ cells ml$^{-1}$), growth rates ($3.25 \pm 0.26$ days compared to $2.85 \pm 0.21$ days) and fatty acid profiles compared to reagent grade glycerol. There were alterations in the carbon partitioning of the microalgae, in addition to changes in cell widths. Cell widths increasing when harvested at stationary phase, compared to reagent grade glycerol (4.88 compared to 4.28 $\mu$m), while chlorophyll (11.38 compared to 6.25 %DW) and carbohydrate contents decreased (17.29 compared to 14.15 %DW respectively). As a result, it can be concluded that this method of contaminant removal meant that crude glycerol may be successfully used for culture of *P. tricornutum*, which may reduce the costs of microalgal culture, depending upon the end use.

**Keywords:** Crude glycerol, mixotrophy, microalgae, biotechnology.

# 1 Introduction

Current production costs of photoautotrophic microalgal biomass have been estimated to be €3.4 $kg^{-1}$ for a theoretical production facility [1]. There are a number of major costs of production, both as capital expenditures (CAPEX) and operating costs (OPEX). OPEX costs include: labour, power and culture medium [2]. While reduction of CAPEX, labour costs or power requirements are beyond the scope of this project, medium selection and optimisation may reduce the costs of biomass production, while maximising cell density and the % dry weight (DW) of the products of interest [3]. One mechanism to maximise the cell density and the products of interest is through mixotrophic or heterotrophic culture of microalgae [4]. For microalgal culture there is a high cost of many medium components, such as nitrogen, phosphate or, in the case of mixotrophic and heterotrophic culture, organic carbon [2]. For example, glucose may represent 80 % of costs of the medium required for heterotrophic culture of *Chlorella prototheciodes* [5]. To reduce the costs of medium components alternative, lower cost, sources may be required. Waste products from other processes are potential sources of different medium components, such as nitrogen [6], phosphate [7], carbon [8] or a combination of several nutrients, such as waste water from recirculated aquaculture systems providing both nitrogen and phosphate [9].

Crude glycerol is a waste product which has received interest as an organic carbon source for a number of thraustochytrid species [8], *C. vulgaris* [10; 11; 12], *C. prototheciodes* [13], *Thalassiosira pseudonana* and a genetically engineered strain of *Phaeodactylum tricornutum* [14]. This interest is due to crude glycerol being a waste product from biodiesel production [15] which is not, currently, economically viable to remove contaminants from this waste product for commercial use [16], even for high value products such as pharmaceuticals or cosmetics [17]. In addition, it poses a potential environmental risk in high volumes [18]. Furthermore, biodiesel can be produced utilising an existing waste stream, waste oil utilised in cooking, which currently represents a 60 % of the costs of sewer and pump cleaning in wastewater treatment [19]. For example, in Scotland approximately 60 million tonnes of biodiesel is produced per year [20] meaning there is a large existing source of crude glycerol.

Unfortunately, crude glycerol from biodiesel production has a high content of contaminants such as heavy metals, methanol and saponified fatty acids, with the saponified fatty acid component identified as a major population growth limiting contaminant in bacterial culture [15]. If this crude glycerol could be utilised to culture microalgae without growth limitation due to contaminant presence, this may represent a method of reducing the costs of culture of species and strains of commercial interest. There are a number of different methods for the removal of contaminants to remove saponified fatty acids, including chemical precipitation using calcium salts, pH adjustment and activated carbon [21]. These contaminant removal methods have been demonstrated to be effective in enhancing the cell density and hydrogen production of the bacterium *Rhodopseudomonas palustris* [21]. If contaminant removal methods could be utilised to develop crude glycerol as a potential organic carbon source for algal culture, which may reduce the costs of utilising mixotrophic production of potential products of interest, such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or fucoxanthin [22].

In this study the growth of the model diatom *P. tricornutum* with reagent grade glycerol is compared to culture with

crude glycerol, both without contaminants removed and using three alternative processes to remove contaminants from the crude glycerol [21].

# 2 Materials and Methods

## 2.1 Utilising crude glycerol as a low cost organic carbon source

The crude glycerol utilised in this study was the waste product of the production of biodiesel from spent chip oil. This biodiesel was produced from a single stage batch trans-esterification process utilising methanol and a sodium hydroxide catalyst. To determine the carbon content of the glycerol, triplicate samples of 10 g of crude glycerol were freeze-dried for 48 hours (Christ Alpha 1-2 LD Plus) and then reweighed to give the volatile content of the sample. These freeze-dried samples were then analysed for carbon and nitrogen content (Costech elemental analyser with acetonitrile as the analytical standard). Initial combustion occurred in a chromium oxide column at 950 °C, reduced using a copper reducing agent at 650 °C and flash combustion at 1800 °C for a total run time of 15 minutes. Further compositional analysis was not conducted as the crude glycerol fraction of biodiesel waste is subject to batch to batch variability, the influences of which were considered to be beyond the scope of this study, which solely aimed to provide preliminary data for the growth of *P. tricorunutum* using this alternative organic carbon source [23].

Three different contaminant removal methodologies were trialled [21]. To remove the contaminants of the crude glycerol using an adjusted pH, between 0-1.2 ml of HCl (12 M) was added to 50 ml of crude glycerol to reach pH 7.0, which had previously been diluted 50 % using ultra-pure water. This mixture was vortexed and centrifuged (2907 g, 5 minutes, Heraeus Multifuge X3FR). Two phases were formed and the lower, glycerol-rich, layer was separated from the free fatty acid layer by use of a serological pipette. Once removed this layer was filtered through a 0.22 $\mu$m PES filter (Millipore express plus Stericup). Calcium precipitation was performed by adding 25 ml of either $CaCl_2$ or $Ca(NO_3)_2$ (0.6 M) to the same volume of undiluted crude glycerol. This mixture was vortexed and filtered using a 0.22 $\mu$m PES filter (Millipore express plus Stericup), with a 50 % additional dilution for the mixture of $CaCl_2$ due to low filtration speed.

Using these organic carbon sources *P. tricornutum* was tested for growth in 50 ml cultures in F/2 + Si [24]. All media tested was iso-carbon (2 g carbon $l^{-1}$). The crude glycerol containing media was made using 11.80 g $l^{-1}$ of 50 % diluted crude glycerol either without contamination removal, contaminants removed using $CaCl_2$ or $Ca(NO_3)_2$ or contaminants removed using a change in pH. These media were compared the F/2+ Si with 5.12 g $l^{-1}$ reagent grade glycerol. Cell densities were quantified by removing 1 ml of a 50 ml culture every Monday, Wednesday and Friday for three weeks. These cultures contained a 10 % (*v/v*) inoculum of mixotrophically cultured *P. tricornutum* obtained from the Culture Collection of Algae and Protozoa (CCAP) in Oban, that had previously been maintained mixotrophically with reagent grade glycerol. The strain identification was: CCAP 1055/1. All cultures were maintained in the conditions previously described [3]. If cell densities did increased across the sampling period then these conditions were subcultured

for biochemical profile analysis. Successful cultures were subcultured into fresh media and triplicate cultures harvested at mid-growth or stationary-phase and the biochemical profile was analysed using the previously described methodology [3].

## 2.2 Data screening and statistical analyses

All data analysis was undertaken in R Studio (V4.02, 2021.09.1). The cell densities of the reagent grade glycerol cultures and pH adjusted crude glycerol cultures were compared using a non-linear least squares modelling approach, fitting a logistic growth model to the cell densities measured. The starting values for the NLS model were derived from a liner model, with the starting carrying capacity derived from the maximum cell density measured across the sampling period. The biochemical profile of all samples were initially tested for normality (Shapiro-Wilk's test) and if normally distributed ($P>0.05$) these were tested for equal variance (Levene's test) and differences between the treatments (ANOVA and *post hoc* Tukey's test). If samples were not normally distributed the differences in treatments were compared using a Kruskal-Wallis test and *post hoc* Dunn's non-parametric comparison and all data presented to 2 decimal places. The fatty acid profiles of the harvested cultures were analysed by placed the profiles into a dissimilarity matrix (Euclidean) utilising the 'dist' function in R studio, without transformation, and comparing the differences between groups analysed using ANOSIM.

# 3 Results

## 3.1 Algal population growth

Culture of *P. tricornutum* with crude glycerol did not lead to an increased cell density compared to the starting inocula for crude glycerol, without contaminant removal, nor either method of calcium precipitation. There was, however, population growth when cultures were grown with crude glycerol which had contaminants removed using a pH change (mean= 4.65 $x10^6$ cells $ml^{-1}$, s.d= 5.33 $x10^5$ cells $ml^{-1}$ for reagent grade cultures compared to mean= 3.8 $x10^6$ cells $ml^{-1}$, s.d= 8.58 $x10^4$ cells $ml^{-1}$ for pH changed crude glycerol cultures when both harvested at stationary phase, Fig: 1).

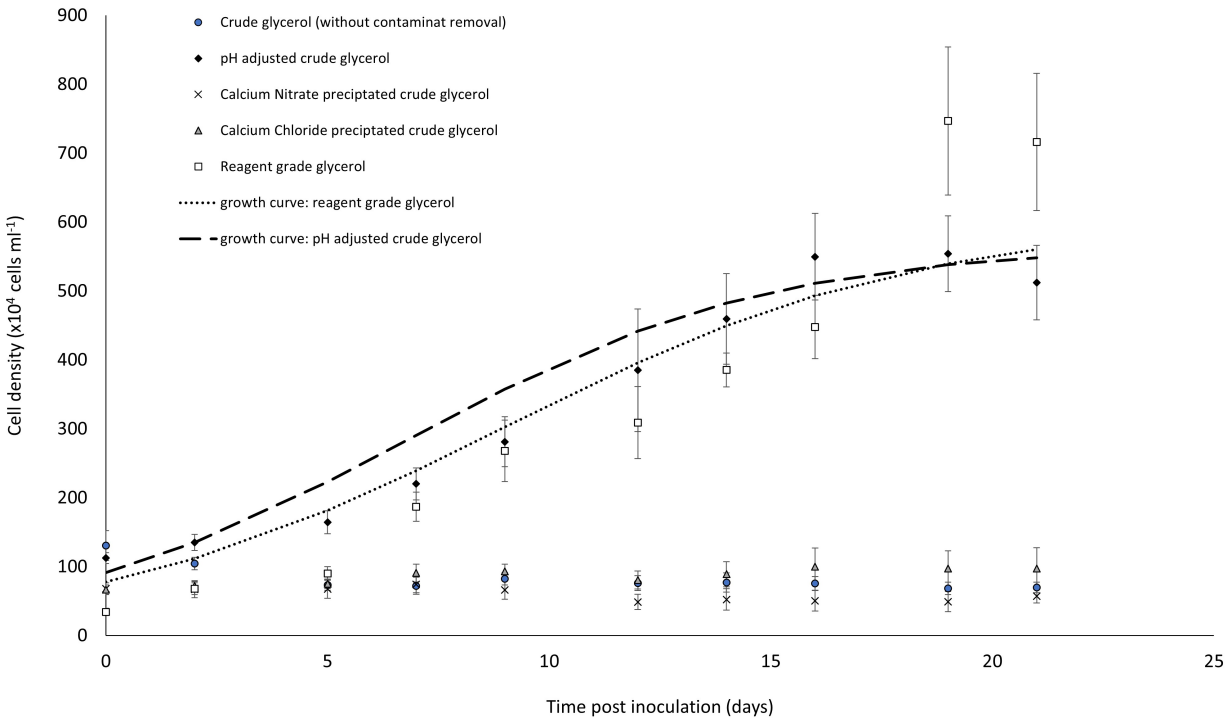


**Fig: 1. Cell densities *P. tricornutum* cultivated with crude glycerol, without contaminant removal (blue circles), pH adjusted (black diamonds), $Ca(NO_3)_2$ precipitated (crosses), $CaCl_2$ precipitated (grey triangles) and reagent grade glycerol (white squares) across the sampling period.**

## 3.2 Biochemical profiles of *P. tricoruntum*

The carbohydrate and chlorophyll contents and the cell widths were normally distributed (Shapiro-Wilks test, W= 0.97048, 0.91264 and 0.94307 respectively, P>0.05) and variances could be considered to be equal (Levene's test, F= 0.4496, 0.3408 and 1.563 respectively, df= 3, P>0.05). The protein, TFA and carotenoid contents were not normally distributed (Shapiro-Wilks test, W= 0.85909, 0.62351 and 0.59314 respectively). There were significant differences in the carbohydrate and chlorophyll contents and the cell widths of the cell (ANOVA, F= 8.865, 9.745 and 40.72 respectively, df= 3, P<0.05), while there were no significant differences in the protein, TFA and carotenoid cultures, irrespective of harvesting time or carbon source (Kruskal-Wallis test, $\chi^2$ = 3.1026, 0.74359 and 7.4615 df = 3, P >0.05 respectively). *Post hoc* Tukey's tests indicated that there were significantly greater carbohydrate contents of cultures harvested at growth phase of reagent grade glycerol, compared to the same carbon source at stationary phase (Fig: 2, Table: 1). Furthermore, stationary phase cultures maintained with reagent grade glycerol also had a greater carbohydrate content than cultures maintained with crude glycerol, harvested at growth phase, although there was no significant difference between the two carbon sources harvested at stationary phase (Fig: 2, Table: 1). Cell widths were lower for cultures harvested in stationary phase, compared to growth, irrespective of glycerol source (post hoc Tukey's test, P<0.05), while cultures grown in crude glycerol and harvested at growth phase also had a greater cell width compared to stationary phase reagent grade glycerol cultures. Cells harvested at stationary phase, when cultured with crude glycerol were also significantly wider than harvesting at stationary phase, when maintained with reagent grade glycerol (Fig: 2, Table: 1). *P. tricornutum* maintained in crude glycerol and harvested at growth phase, also had a significantly lower chlorophyll content compared to cultures in

reagent grade glycerol, irrespective of harvesting time although, this was not the case for reagent grade glycerol cultures harvested during stationary phase (Fig: 2, Table: 1). When comparing reagent grade glycerol cultures against cultures grown on crude glycerol, the fatty acid profile did not significantly explain the variation in the groupings (ANOSIM, R= 0.11, P>0.05).

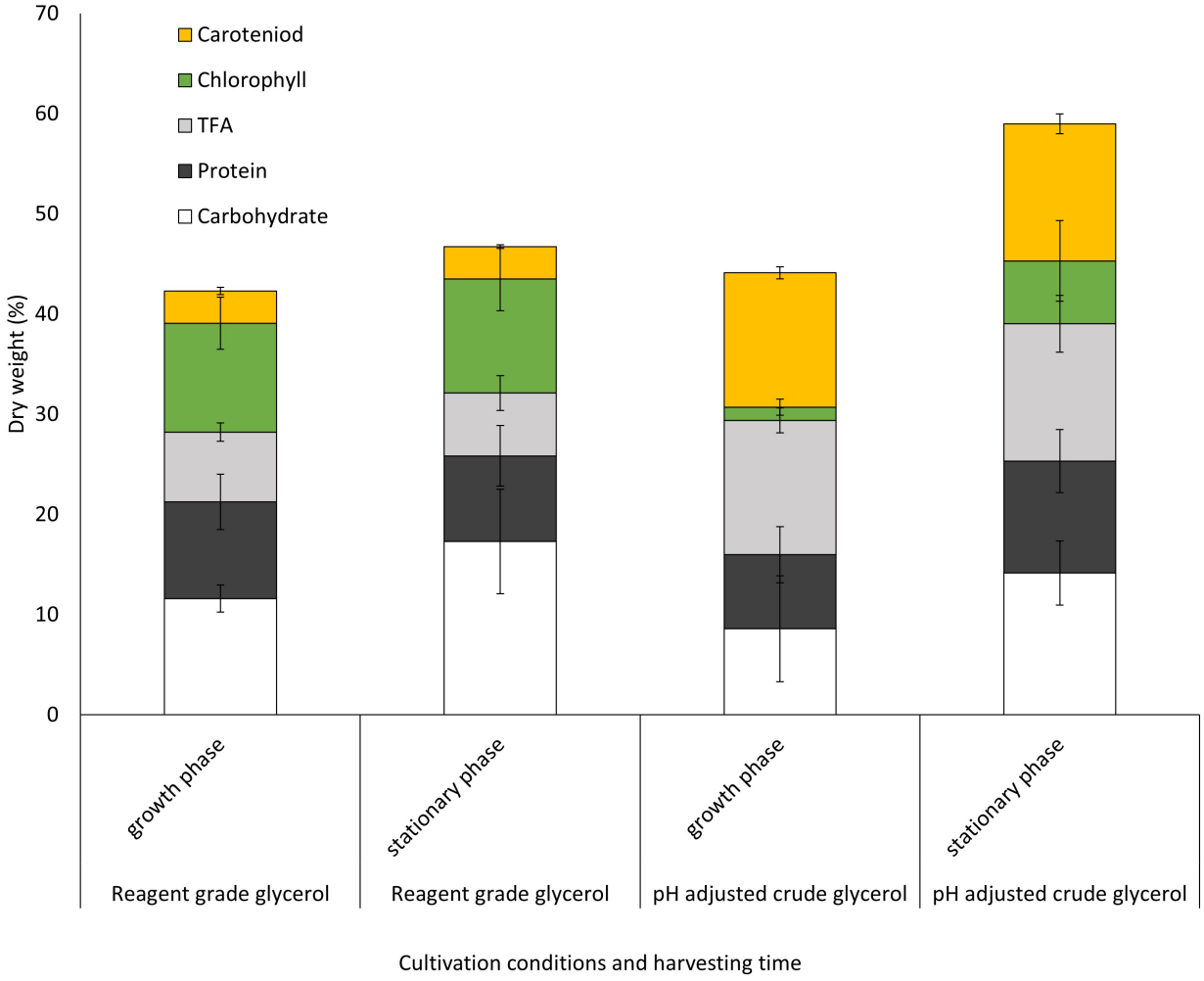


**Fig: 2. Biochemical profile of reagent grade and pH changed crude glycerol cultures of *P. tricornutum*.**

**Table 1: Biochemical profiles of reagent grade and reagent grade glycerol cultures, harvested at the indicated culture phase and the logistic growth parameters derived. All values are ± standard deviation.**

| | Carbohydrate (% DW) | Protein (% DW) | TFA (% DW) | Cell width (% DW) | Chlorophyll (% DW) | Carotenoid (% DW) |
|---|---|---|---|---|---|---|
| **Reagent grade glycerol: growth phase** | 11.60 ± 1.35 | 9.65 ± 2.75 | 6.97 ± 0.91 | 4.80 ± 0.029 | 10.85 ± 2.60 | 3.23 ± 0.38 |
| **Reagent grade glycerol: stationary phase** | 17.29 ± 5.20 | 8.55 ± 3.02 | 6.28 ± 1.74 | 4.28 ± 0.36 | 11.38 ± 3.17 | 3.23 ± 0.18 |
| **pH adjusted crude glycerol: growth phase** | 8.59 ± 5.27 | 7.39 ± 2.81 | 13.41 ± 1.25 | 5.26 ± 0.31 | 1.33 ± 0.81 | 13.41 ± 0.60 |
| **pH adjusted crude glycerol: stationary phase** | 14.15 ± 3.20 | 11.19 ± 3.14 | 13.69 ± 2.83 | 4.88 ± 0.14 | 6.25 ± 4.03 | 13.69 ± 1.00 |

| **Growth parameters** | **$\mu$ max** | **generation time (days)** | **carrying capacity x10 $^4$ cells ml$^{-1}$** | **Residual standard error** | **degrees of freedom** |
|---|---|---|---|---|---|
| **Reagent grade glycerol** | 0.21295 ± 0.01824 | 3.25 ± 0.26 | 603.49 ± 24.35 | 56.65 | 117 |
| **pH adjusted crude glycerol** | 0.24294 ± 0.01931 | 2.85 ± 0.21 | 565.97 ± 15.18 | 53.52 | 117 |

# 4 Discussion

*P. tricornutum* was tested for population growth utilising media containing crude glycerol following contaminant removal by one of a number of different methodologies. This resulted in successful population growth for *P. tricornutum* when cultured with crude glycerol that had contaminants removed by pH change, while there was only a slight reduction in cell density at stationary phase when crude glycerol was utilised as the source of organic carbon compared to reagent grade glycerol. Furthermore, there were no significant changes in the TFA or protein content compared to reagent grade glycerol cultures, while there was a significant changes in the cell widths, chlorophyll and carbohydrate contents.

## 4.1 Utilisation of crude glycerol as a low cost organic carbon source

A low cost alternative carbon source which utilises an existing waste product is an important step to reduce the costs of microalgal culture, as the addition of an organic carbon source can be high [5]. When cultures of *P. tricornutum* were maintained in the presence of crude glycerol, which had contaminants removed using a change in the pH of the glycerol, the population growth curve was comparable to culture with reagent grade glycerol (Fig: 1). By contrast, no other contaminant removal method resulted in cell densities greater than the starting inocula, nor did crude glycerol without contaminant removal. When utilising calcium precipitation to remove contaminants from crude glycerol it was necessary to increase the dilution to four times to be able to filter the sample through a 0.2 $\mu$m filter. While previous studies have shown successful bacterial culture through use of activated carbon, this method of contaminant removal was not tested, due to the costs of using activated carbon [21]. The lack of population growth in *P. tricornutum* when cultured with crude glycerol with contaminants removed by calcium precipitation is in contrast to work in *R. palustris* which resulted in an increase in bacterial population growth [21]. Reasons for this are currently unclear, but may suggest that the presence of saponified fatty acids are not the only population limiting contaminant in the crude glycerol and a change in the pH also removes these other factors sufficiently, while calcium precipitation did not.

Successful growth with crude glycerol has been observed with a range of other microalgal species such as: *Chlorella vulgaris, Botryococcus braunii* and *Scenedesmus* sp. [25] and *T. pseudonana* [26]. For these species growth inhibition at higher concentrations of crude glycerol, without contaminant removal have been observed [11], although at varying concentrations depending upon the strain and source of crude glycerol. Therefore, future work should quantify growth of *P. tricornutum* on different concentrations of crude glycerol and compare these to culture with a iso-carbon reagent grade glycerol concentration. Understanding of the strain and source specific effects on the growth and biochemical profiles of microalgae are limited as conditions in some studies have other factors within their methodologies which render direct comparison challenging, potentially masking inhibitory effects. For example, culture of *C. vulgaris, B. braunii* and *Scenedesmus* spp. was not compared to culture with reagent grade glycerol [25]. Similarly, lack of comparison between iso-carbon reagent grade and crude glycerol cultures; 0-10 g $l^{-1}$ crude glycerol compared to 25 g $l^{-1}$ reagent grade glycerol for culture of *C. vulgaris* [10], makes direct comparison challenging. Different cultivation modes also make direct comparison challenging, such as photoautotrophic culture with additional carbon dioxide of *C. vulgaris* in an air uplift bioreactor compared to mixotrophic culture with crude glycerol [12]. The difficulty of direct comparison is due to mixotrophic alteration of algal population growth and carbon partitioning, compared to photoautotrophy [27].

The biochemical profiles of *P. tricoruntum* cells harvested at the same stage of culture were not significantly different when comparing protein, TFA, fatty acid profiles or carotenoid contents, while there were significant differences in the cell width, carbohydrate and chlorophyll content, when comparing reagent grade glycerol cultures with crude glycerol cultures. There was a decrease in the chlorophyll contents for cultures maintained in the pH adjusted crude glycerol alongside an increased cell width (Fig: 2, Table: 1). This increase is similar to *T. pseudonana* which had an decreased chlorophyll content, when comparing between growth phase cultures or stationary phase cultures of crude and reagent

grade glycerol [26]. This may indicate that there are a decreased number of plastids in these conditions and a shift in the carbon partitioning away from photoautotrophy in crude glycerol containing cultures, potentially as a stress response to those contaminants in the medium that were not removed by the pH change, which has been shown to occur in *Dunaliella salina* [28]. There was an increase in the carbohydrate contents of *P. tricornutum* harvested at growth phase, compared to stationary phase, corroborating previous studies which have indicated that at stationary phase this strain accumulates lipids in preference to carbohydrates [3; 29]. Carbohydrate storage in the reagent grade glycerol may be due to photosynthesis driven accumulation of chrysolaminarin, compared to lipid accumulation [30]. Previous observations have quantified glucose as contributing 90% of biomass carbon accumulated in strain CCMP632 [31], however; further work is necessary to elucidate the alterations in the carbon partitioning of glycerol in this species.

It can be concluded that culture with crude glycerol, which had contaminant removal by pH change, may be a viable alternative to reagent grade glycerol for cultures of this strain of *P. tricornutum*. This is advantageous due to the lower cost of crude glycerol [16]. Further work is necessary to test the viability of this medium component at larger culture scales, in addition to the potential contaminants which have small, or non-significant effects upon algal culture, but remain within the media and may have negative effects upon alternative end products. For example, higher contents of heavy metals may have a negative effect if the *P. tricornutum* cultures were utilised directly as aquaculture feed [32]. Depending upon the endpoint useage of the microalgal biomass, there is likely to be a trade-off between the maximum productivity attainable for an algal product, for the minimum price. For example, if the end-point useage requires a greater carbohydrate content then reagent grade glycerol may be required, however; if this is not necessary, then crude glycerol may be a low cost alternative [33].

## 4.2 Conclusions

*P. tricornutum* was tested for its capacity to growth with crude glycerol: without contaminant removal, contaminants removed using pH change or contaminants removed using precipitation with either calcium nitrate of calcium chloride. Population growth was only observed when reagent grade glycerol, or crude glycerol from which had contaminants had been removed using a pH change methodology. The biochemical profile of these cultures was not significantly different compared to reagent grade glycerol cultures, with the exception of a significantly increased cell width and decreased chlorophyll and carbohydrate contents when harvested at stationary phase of culture. This suggests that crude glycerol may be a viable source of organic carbon to maximise culture densities, depending upon the intended use of the biomass, for a reduced cost.

# Conflict of Interest and Ethical statement

No conflicts, informed consent, or human or animal rights are applicable to this study. The authors declare that there are no conflicts of interest. This work has been funded by the European Social Fund and the Scottish Funding Council.

# CRediT authorship contribution statement

JKPS, ADH, LM and JGD conceived the study design and analysis. JKPS drafted the manuscript and all authors edited the manuscript. All authors have read and approved the final version.

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