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Research Paper

# Synergistic carbon metabolism in a fast growing mixotrophic freshwater microalgal species *Micractinium inermum*

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ABSTRACT

In recent years microalgae have attracted significant interest as a potential source of sustainable biofuel. Mixotrophic microalgae are able to simultaneously photosynthesise while assimilating and metabolising organic carbon. By combining autotrophic and heterotrophic metabolic pathways biomass productivity can be significantly increased. In this study, acetate-fed mixotrophic *Micractinium inermum* cultures were found to have a specific growth rate 1.74 times the sum of autotrophic and heterotrophic growth. It was hypothesised that gas exchange between the two metabolic pathways within mixotrophic cultures may have prevented growth limitation and enhanced growth. To determine the extent of synergistic gas exchange and its influence on metabolic activity, dissolved inorganic carbon (DIC), dissolved oxygen (DO) and photosynthesis and respiration rates were measured under different trophic conditions. A 32.7 fold and 2.4 fold increase in DIC and DO concentrations, relative to autotrophic and heterotrophic cultures respectively, were coupled with significant increases in rates of photosynthesis and respiration. These data strongly support the hypothesis of mixotrophic gas exchange within *M. inermum* cultures. In addition to enhanced growth, this phenomenon may provide reductions in aeration and oxygen stripping costs related to microalgae production.

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## 1. Introduction

Microalgae derived biofuels have the potential to meet a significant fraction of global transport fuel demand, enabling a reduction in our dependency on finite fossil fuels and their associated greenhouse gas emissions. Although microalgae have similar photosynthetic machinery to higher plants, their simpler cellular structure and planktonic mode of growth allow efficient access via diffusion to $CO_2$ and other nutrients, enabling high biomass productivities. Furthermore, oleaginous microalgae accumulate significant proportions of their biomass as lipids (typically 20–50% compared to <5% for oil palm); lipid being the desired feedstock for biodiesel production [1]. Consequently, compared to terrestrial crop biofuel feedstocks such as soybean or sugarcane, microalgae have orders of magnitude higher land use efficiencies [2]. In addition, microalgae can be grown on non-arable degraded land using wastewater as a nutrient source [3]. These attributes are particularly important because, unlike first generation biofuels, they allow extensive microalgal cultivation while avoiding the economic and environmental issues associated with food production competition.

Unfortunately, the numerous advantages of microalgal biofuels are matched by several technical challenges that must be overcome to reach full environmental and economic feasibility. One such challenge is achieving a high enough productivity to drive down processing and harvesting costs, which, due to the microscopic and aqueous nature of microalgae, are considerably higher than terrestrial crops [3]. Biomass productivity, expressed, for example, in units of grams of dry cells per litre of culture per day, depends on both cell density and specific growth rate [4]. Photoautotrophic (henceforth referred to as autotrophic) algae rely entirely on solar energy for growth, consequently cell density is inversely proportional to light penetration and as cell density increases specific growth rate declines due to self-shading [5]. Achieving a high cell density and biomass productivity can therefore be limited by light availability. For large-scale cultivation systems, photosynthetic autotrophic growth is usually considered as the dominant mode for engineering design purposes. For this mode of growth, reactors and ponds are designed to ensure adequate access to sunlight for photosynthesis, with operating cell densities maintained at a reasonably low level to prevent excessive shadowing of other cells. On the other hand, heterotrophic cultivation of microalgae by promoting aerobic respiration on an organic carbon source enables biomass production to be maintained during the night hours, and at higher cell densities due to light independent growth. Cultivation using both modes of carbon metabolism therefore offers the potential to maximise productivity; particularly when a source of cheap organic carbon is available such as acetate in the effluent of anaerobic digestors [6].

Mixotrophic algae are capable of simultaneously photosynthesising while assimilating and metabolising organic carbon as both a carbon and energy source. This mode of growth reduces dependency on light penetration, enabling higher cell densities than autotrophy, while using considerably less organic material per unit of biomass than dark heterotrophic growth [7]. Mixotrophic growth can enable significantly enhanced biomass productivity, which in turn leads to enhanced lipid productivity [5]. These characteristics make mixotrophy a promising alternative strategy for microalgae biofuel production, especially when coupled with the use of waste organic carbon sources [8].

A frequently overlooked challenge facing microalgal biofuel commercialisation is $CO_2$ supply. Autotrophic algal growth is highly dependent on the availability of $CO_2$, which provides the main source of elemental carbon for the production of new biomass and energy carrying molecules. Carbon dioxide is freely available in the atmosphere, but at a low concentration of around 0.04% which limits the diffusive mass transfer of $CO_2$ into an algal culture. In order to reach economically feasible productivity rates, this mass transfer limitation to autotrophic growth can be removed by sparging cultures with external sources of concentrated $CO_2$. Anthropogenic $CO_2$ sources such as waste flue gases from power plants are envisaged to meet the requirement for large scale microalgae production. However, providing sufficient $CO_2$ to produce a quarter of US annual transport fuel demand (roughly 227 billion litres of biodiesel) would require harnessing a large proportion (36–56%) of total $CO_2$ emissions from all US power plants [9,10]. Furthermore, in the US, without an extensive and costly infrastructure of $CO_2$ capture and transportation, only a limited number of these concentrated emission sources are within range of areas suitable for large scale algal cultivation. Consequently the availability and cost of supplying $CO_2$ is a significant bottleneck for large scale microalgae production and further research is needed to reduce reliance on external concentrated $CO_2$ sources [10,11].

Mixotrophic growth could mitigate this problem by providing an endogenic source of $CO_2$ through the respiration of organic carbon [12]. One of the earliest experiments comparing trophic modes in the green alga, *Chlorella regularis*, discovered that the two metabolic pathways, photosynthesis and heterotrophic respiration, can act non-competitively under mixotrophic growth [13]. Consequently the mixotrophic specific growth rate should equate to the sum of the autotrophic and heterotrophic growth rates. This can be expressed as a specific growth rate ratio (Equation (1));

$$\frac{\mu_M}{\mu_A + \mu_H} = 1 \quad (1)$$

where $\mu_M$, $\mu_A$, $\mu_H$, equate to mixotrophic, autotrophic and heterotrophic specific growth rates respectively. Subsequent studies have reported a similar relationship in *Chlorella vulgaris, Haematococcus pluvialis, Chlamydomonas humicola*, but not in *Scenedesmus acutus* [14–17]. The species specific nature of non-competitive mixotrophic growth is largely influenced by both the degree to which organic carbon assimilation inhibits the production of chlorophyll and the degree to which illumination inhibits the production of organic carbon uptake enzymes [12,16]. However, conformity to this ratio is also governed by the aeration conditions under which the cultures are grown. Martinez and Orus [15] reported that when the *C. vulgaris* cultures were aerated with 2% $CO_2$-air the ratio was recorded as 1.00, in line with earlier aerated studies. However when the cultures were aerated with air or grown under non-aerated conditions this ratio increased to 1.10 and 1.28 respectively. A possible explanation for this phenomenon is

that under non-aerated conditions autotrophic and heterotrophic cultures are limited by $CO_2$ and $O_2$ respectively. In mixotrophic conditions the activity of both metabolic pathways could enable synergistic rather than non-competitive growth, with the assimilation and metabolism of organic carbon providing an endogenic source of $CO_2$ to fuel photosynthesis, which in turn provides an enriched source of $O_2$ for respiration. This synergistic effect could reduce gaseous growth limitations and enhance growth. On the other hand it is possible that the assimilation of photosynthetically derived $O_2$ by respiration could prevent oxygen accumulation. This principle could be extended to cultures containing communities of two or more algal species.

This work investigated the growth of a locally isolated strain of *Micractinium inermum* under autotrophic, heterotrophic and mixotrophic conditions. The objectives of the study were (1) to investigate the specific growth rate ratio of *M.inermum* with different organic carbon sources (2) to determine the degree of $CO_2$ and $O_2$ limitation in autotrophic and heterotrophic *M.inermum* cultures respectively, and (3) to investigate the capability of mixotrophic growth to reduce these growth limitations via synergistic gas exchange between the two metabolic processes.

## 2. Materials and methods

### 2.1. Identification of the microalga

A mixotrophic green algae species was isolated from Weston Park Pond, Sheffield, UK. Total DNA was extracted from lyophilised cells from both mixotrophic and autotrophic cultures following the procedure outlined by Chen et al. [18]. Subsequently small subunit (SSU) RNA gene sequences for 18S, ITS1, 5.8S, and ITS2 were amplified by polymerase chain reaction (PCR) using primers detailed in Huss et al. [19] and Hoshina and Fuiwara [20]. Purified PCR products were sequenced (Eurofins MWG Operon) in forward and reverse directions using cycle sequencing technology on an ABI 3730XL sequencing machine. SSU sequences were assembled using SeqMan Pro software (12.0.0222, DNASTAR, Inc) and compared with existing sequences on the NCBI GenBank database via BLAST. The closest alignment match was *Micractinium inermum (NLP-F014)* (accession number KF597304) with a 99% similarity. Consequently the species was identified as *Micractinium inermum*. The new sequences for the strain used in this work were deposited in the NCBI database under the accession number KM114868.

### 2.2. Growth conditions

*M. inermum* was grown in 3N-BBM + V medium (www.ccap.ac.uk); with the addition of HEPES buffer (0.05 M). Initial pH was adjusted to 6.5. For heterotrophic and mixotrophic growth treatments, the base medium was modified with the addition of 1.2 g $l^{-1}$ (14.6 mM) sodium acetate or 1.2 g $l^{-1}$ (6.67 mM) D-glucose.

*M. inermum* was inoculated at 1% (v/v) from a mixotrophic subculture into 250 ml Erlenmeyer flasks containing 100 ml of fresh medium. Inoculum cultures were diluted to a cell density of $5.5 \times 10^5$ $ml^{-1}$. The flasks were placed in a temperature controlled room (25 °C ± 2) and agitated daily. Autotrophic and mixotrophic cultures were grown under continuous illumination with a light intensity of 91 ± 7 μmol $m^{-2}$ $s^{-1}$. Heterotrophic cultures were wrapped in foil and incubated in the dark in the 25 °C room. Experiments investigating dissolved oxygen and inorganic carbon concentrations were carried out using a fed-batch approach, replacing the extracted culture volume with fresh medium (5 ml). All flask experiments were carried out in quintuplicate. In aerated experiments, cultures were grown in specially designed 130 ml tubular air loop miniature bioreactors fitted with ceramic diffusers (design detailed in Fig. S1 in the supplementary material, [21]), containing 100 ml of medium. The cultures were grown in a temperature controlled room (25 °C ± 2). Autotrophic cultures were aerated under two treatments of aseptic filtered (0.22 μm) 5% $CO_2$ (balance air) and atmospheric air (0.04% $CO_2$) with a flow rate of 5 $cm^3$ $min^{-1}$. Heterotrophic cultures were aerated under two treatments of aseptic filtered (0.22 μm) 100% $N_2$ (0% $O_2$) and atmospheric air (21% $O_2$) with a flow rate of 10 $cm^3$ $min^{-1}$. Additionally, air was pumped through a perforated silicone tube ring at the base of reactors for 1 min every 3 h at a flow rate of 120 cc/min in order to resuspend cells accumulated in dead zones of the reactor (Fig. S1). Autotrophic cultures were grown under continuous illumination with a light intensity of 77 ± 9 μmol $m^{-2}$ $s^{-1}$. Heterotrophic cultures were grown in the dark. Aerated experiments were also carried out in a fed-batch approach in order to maintain the liquid volume in the bioreactor (1 ml daily replacement). Aerated experiments were carried out in quadruplicate.

### 2.3. Determination of biomass concentration, growth rate and productivity

Dry weight was determined gravimetrically by oven drying $dH_2O$ washed algal pellets at 50 °C for 24 h. Cell density was measured using a haemocytometer as outlined in Guillard and Sieracki [22]. Growth was monitored daily by using a UV–Vis Unicam Helios α spectrophotometer to quantify the optical density of the culture at 600 nm ($OD_{600}$). Each plastic cuvette was agitated before a reading was taken to ensure homogeneous cell dispersal. Cultures were checked daily through microscopy observation for bacterial contamination. $OD_{600}$ was calibrated with cell density and dry weight for each trophic condition and carbon source. Calibration equations (Table S1), calculations of specific growth rate (μ, Equation S(1)), and maximum biomass productivity ($Pr_{max}$, Equation S(2)) are outlined in the supplementary material.

### 2.4. Chlorophyll analysis

Prior to analysis, cells were freeze dried for 48 h and ground with a glass rod. Freeze dried samples were resuspended in $dH_2O$ and sonicated for 30 s (10 kHz) followed by chlorophyll extraction with 90% acetone as described by Jeffery and Humphrey [23]. All analyses were carried out in biological triplicate.

### 2.5. Dissolved inorganic carbon and dissolved oxygen measurements

Dissolved oxygen (DO) was measured using a Thermo Scientific Orion 3-Star Plus™ Dissolved Oxygen probe. A 5 ml aliquot of culture was transferred to a magnetically stirred 10 ml flask. The probe was inserted and a stable reading was taken at 20 °C ± 1. At the pH range of 6.5–7.5 measured during cultivation, dissolved inorganic carbon (DIC) analysis provided a reliable measurement of the dissolved inorganic carbon species available for photosynthetic assimilation including dissolved $CO_2$ and bicarbonate. DIC was measured using an acid stripping method adapted from Hodson et al. [24]. A 5 ml aliquot of culture was filtered through a Whatman™ 1.2 μm glass microfiber filter to remove algae cells. The supernatant was transferred to a closed, $CO_2$ purged, acidifying system (hand-built via a 3-Gang Luer™ Stopcock Manifold connecting 4 syringes). Within the manifold a 15 ml aliquot of air was passed through a $CO_2$ scrubber (60 ml syringe containing 35 ml of soda lime granules) and transferred to a syringe containing 2 ml of 3 M HCl. The mixture was then drawn into the sample and immediately sealed. The sample syringe was agitated for 1 min and subsequently left to stand for 2 min. The 15 ml headspace was transferred to a dry 20 ml syringe then injected into a PP Systems EGM4 IR $CO_2$ gas analyser. A stable reading was taken. The DIC was calculated from a calibration curve using five $Na_2CO_3$ solution standards (0, 0.125, 0.25, 0.5 and 1 mM C).

### 2.6. Net $O_2$ evolution

Photosynthetic and respiration rates were determined daily using a Hansatech Clark-type oxygen electrode connected to a DrDaq data logger. After calibration with sodium dithionite, a 2 ml aliquot of culture of known cell density was transferred to a magnetically stirred and temperature controlled (25 °C) electrode chamber. The net $O_2$ evolution was continuously monitored under illumination (68 μmol $m^{-2}$ $s^{-1}$) for a 6 min period followed by 6 min in the dark. Rate of $O_2$ evolution/consumption under light and dark conditions equates to rate of net photosynthesis and respiration respectively. The gross photosynthesis rate was calculated from the sum of net photosynthesis and respiration, i.e., assuming that respiration rate was the same in the light and the dark.

### 2.7. Statistics

All results are expressed as mean values (±standard deviation, SD). The effect of growth conditions on measured observations (inter-treatment variation) were analysed using one-way analysis of variances (ANOVAs) following a linear model. The effect of time on measured observations within a growth condition (intra-treatment variation) were analysed using repeated measures nested ANOVA following a linear mixed effects model (nlme package). Data that did not meet the assumptions of parametric analysis were log transformed before analysis. If analysis indicated a significant effect, the significance of differences between growth conditions (inter-treatment) and time points within a treatment (intra-treatment) were measured using Tukey Honestly Significant Differences (HSD) or Tukey Multiple Comparison using General Linear Hypothesis function (multicomp package) respectively. All analyses were carried out with the statistical software R v.3.1.2 [25].

## 3. Results

### 3.1. Effect of trophic mode on M. inermum growth and chlorophyll content

The effect of organic carbon addition on the growth of *M. inermum* was investigated in a non-aerated batch culture experiment. The specific growth rate (μ) and maximum biomass productivity ($Pr_{max}$) of *M. inermum* under autotrophic, mixotrophic and heterotrophic conditions are compared and summarised in Table 1. As demonstrated in Fig. 1, growth was strongly influenced by the addition of both acetate and glucose (ANOVA F = 1839, d.f 3,17, $p < 0.001$). Autotrophic cultures grew slowly at a linear rate, achieving a specific growth rate of 0.38 $d^{-1}$ ± 0.04 and a $Pr_{max}$ of 10.55 mg $l^{-1}$ $d^{-1}$ ± 0.55. Under heterotrophic conditions *M. inermum* grew exponentially at μ of 0.87 $d^{-1}$ ± 0.07 and 1.40 $d^{-1}$ ± 0.09 for acetate and glucose respectively and achieved a maximum productivity more than 3 fold higher than autotrophic cultures (Table 1). The growth rate of *M. inermum* under mixotrophic conditions was significantly higher than the sum of autotrophic and heterotrophic growth. Mixotrophic cultures achieved specific growth rates more than 5 fold higher and a $Pr_{max}$ nearly 40 fold higher than autotrophic cultures (Table 1). In this study, the specific growth rate ratios (Equation (1)) were 1.74 and 1.34 for acetate and glucose respectively (Table 1).

The chlorophyll content of *M. inermum* cultures under different trophic conditions were analysed during the exponential phase of growth (Table 2). The addition of both acetate and glucose caused a significant decrease in relative chlorophyll production compared to the autotrophic control. This decrease was considerably greater in mixotrophic and heterotrophic glucose supplemented cultures, which were found to have 89% and 97% less chlorophyll (mg $g^{-1}$) respectively compared to autotrophic cultures. In order to avoid photosynthetic limitation, acetate was used in subsequent experiments rather than glucose. The other important reason for focusing on acetate in this study is that this carbon source is far more likely to be freely available for large scale cultivation systems, most notably in the soluble fraction of the liquor produced by anaerobic digesters.

The chlorophyll content of acetate supplemented mixotrophic and heterotrophic cultures was also analysed at the start and end of the stationary phase. Due to slow growth, autotrophic cultures did not reach their stationary phase and continued to grow linearly after 70 days of cultivation; however, results are included for comparison. The chlorophyll content of autotrophic and heterotrophic cultures did not change significantly throughout the growth cycle. Mixotrophic cultures displayed the most variability over time, initially decreasing from the exponential to initial stationary

**Table 1 – Comparison of the effect of light and organic carbon on specific growth rate of mixotrophic species.**

| Species | Growth condition | Light intensity ($\mu$mols $m^{-2}$ $s^{-1}$) | Organic Carbon | Aeration | $\mu$ ($d^{-1}$) | $\frac{\mu_M}{\mu_A+\mu_H}$ | $Pr_{max}$ (mg $l^{-1}$ $d^{-1}$) | Source |
|---|---|---|---|---|---|---|---|---|
| ***M. inermum*** | **Autotrophic** | **91** | **None** | **Shaken daily** | **$0.38 \pm 0.04^{a}$** | – | **$10.55 \pm 0.55^{a*}$** | This study |
| " | **Mixotrophic** | **91** | **NaAc (1.2 g $l^{-1}$)** | **Shaken daily** | **$2.18 \pm 0.07^{b}$** | – | **$351.50 \pm 7.18^{b*}$** | " |
| " | **Heterotrophic** | – | **NaAc (1. g $l^{-1}$)** | **Shaken daily** | **$0.87 \pm 0.07^{c}$** | **1.74** | **$42.04 \pm 0.40^{c*}$** | " |
| " | **Mixotrophic** | **91** | **Glucose (1.2 g $l^{-1}$)** | **Shaken daily** | **$2.46 \pm 0.04^{b}$** | – | **$373.37 \pm 23.10^{d*}$** | " |
| " | **Heterotrophic** | – | **Glucose (1.2 g $l^{-1}$)** | **Shaken daily** | **$1.40 \pm 0.09^{e}$** | **1.37** | **$32.70 \pm 1.75^{e*}$** | " |
| *H. pluvialis* | Autotrophic | 20 | None | Shaken daily | 0.32 | – | – | [14] |
| " | Mixotrophic | 20 | NaAc (0.6 g $l^{-1}$) | Shaken daily | 0.57 | – | – | " |
| " | Heterotrophic | – | NaAc (0.6 g $l^{-1}$) | Shaken daily | 0.18 | 1.14 | – | " |
| *C. vulgaris* | Autotrophic | 70.2 | None | Shaken cont. + 2% $CO_2$ | 2.64 | – | – | [16] |
| " | Mixotrophic | 70.2 | Glucose (5 g $l^{-1}$) | Shaken cont. | 4.75 | – | – | " |
| " | Heterotrophic | – | Glucose (5 g $l^{-1}$) | Shaken cont. | 2.35 | 0.95 | – | " |
| *S. acutus* | Autotrophic | 70.2 | None | Shaken cont. + 2% $CO_2$ | 1.46 | – | – | " |
| " | Mixotrophic | 70.2 | Glucose (0.5 g $l^{-1}$) | Shaken cont. | 1.15 | – | – | " |
| " | Heterotrophic | – | Glucose (0.5 g $l^{-1}$) | Shaken cont. | 0.96 | 0.48 | – | " |
| *C. vulargis* | Autotrophic | 150 | None | Shaken | $0.85 \pm 0.02$ | – | – | [15] |
| " | Mixotrophic | 150 | Glucose (5 g $l^{-1}$) | Shaken | $2.48 \pm 0.09$ | – | – | " |
| " | Heterotrophic | – | Glucose (5 g $l^{-1}$) | Shaken | $1.08 \pm 0.01$ | 1.28 | – | " |
| " | Autotrophic | 150 | None | Shaken + 2% $CO_2$ | $1.95 \pm 0.09$ | – | – | " |
| " | Mixotrophic | 150 | Glucose (5 g $l^{-1}$) | Shaken + 2% $CO_2$ | $3.16 \pm 0.07$ | – | – | " |
| " | Heterotrophic | – | Glucose (5 g $l^{-1}$) | Shaken + Air | $1.2 \pm 0.05$ | 1 | – | " |
| *C. humicola* | Autotrophic | 70 | None | None | $0.21 \pm 0.04$ | – | – | [17] |
| " | Mixotrophic | 70 | NaAc (0.82 g $l^{-1}$) | None | $1.66 \pm 0.24$ | – | – | " |
| " | Heterotrophic | – | NaAc (0.82 g $l^{-1}$) | None | $0.78 \pm 0.21$ | 1.68 | – | " |

Values denoted by a different letter at each data point differ significantly at $p < 0.01$ by (*denoted log transformed) one-way ANOVA and Tukey analysis. Information in bold is from this study.

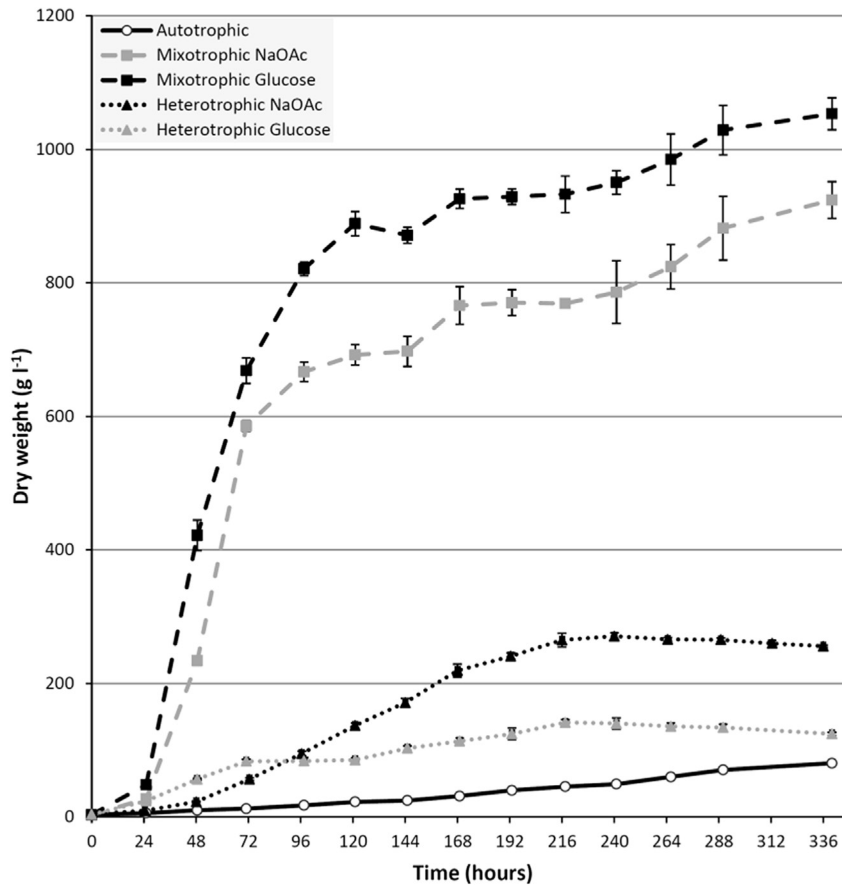


**Fig. 1 – Growth curve of *M. inermum* under autotrophic, heterotrophic (sodium acetate or glucose) and mixotrophic (sodium acetate or glucose) conditions. Experiment carried out in batch flask cultures. Bars represent standard deviation.**

phase, then increasing significantly ($p < 0.05$) at the end stationary phase to a similar (i.e. not significantly different) content to the 70 day old autotrophic cultures (Table 2).

### 3.2. *Gaseous limitation in autotrophic and heterotrophic cultures*

The beneficial effects of mixotrophic gas exchange would be apparent only if, under non-aerated flask conditions, autotrophic and heterotrophic growth rates are limited by $CO_2$ and $O_2$ availability. In order to determine the degree of this gaseous mass transfer limitation on the growth of *M. inermum*, cultures were grown in aerated miniature bioreactors with different gas concentrations: autotrophic cultures were grown under air (0.04% $CO_2$) and 5% $CO_2$ (balance air) aeration; acetate supplemented heterotrophic cultures were aerated with $N_2$ (0% $O_2$) and air (21% $O_2$). $N_2$ aeration was used as a control in heterotrophic cultures to avoid oxygenation of the culture while mirroring the mixing effect of aeration used in the air aerated treatment.

Autotrophic growth was significantly enhanced under both air ($\mu = 1.38\ d^{-1} \pm 0.02$, $Pr_{max} = 80.97\ mg\ l^{-1}\ d^{-1} \pm 23.10$) and 5% $CO_2$ aeration ($\mu = 2.39\ d^{-1} \pm 0.04$, $Pr_{max} = 841.20\ mg\ l^{-1}\ d^{-1} \pm 86.64$), as demonstrated in Fig. 2A. $CO_2$ aeration led to a 6.3 fold increase in μ and 80 fold increase in productivity compared to non-aerated flask cultures (Table 1). There was a significant difference between both μ (ANOVA $F = 699.11$, d.f 1,6, $p < 0.001$) and $Pr_{max}$ (ANOVA $F = 287.5$, d.f 1,6, $p < 0.001$) for air and $CO_2$ aerated cultures.

Under heterotrophic cultivation, air aeration (21% $O_2$) led to 49% higher μ (ANOVA $F = 820.57$, d.f 1,6, $p < 0.001$) and 91% higher $Pr_{max}$ (ANOVA $F = 1982.7$, d.f 1,6, $p < 0.001$) compared to $N_2$ aeration (0% $O_2$) (Fig. 2B). After 168 h growth, the dry weight of air aerated culture peaked at 249 mg $l^{-1}$. This compares to the non-aerated heterotrophic flask culture which reached a dry weight of 240 mg $l^{-1}$ after 192 h and then continued to grow peaking at 271 mg $l^{-1}$ after 240 h (Fig. 1). The slower growing $N_2$ aerated culture reached 191 mg $l^{-1}$ after 240 h growth. The $Pr_{max}$ of air aerated cultures was 97% higher than flask cultures (ANOVA $F = 224.71$, d.f 1,7, $p < 0.001$), but surprisingly the μ of air aerated cultures ($0.74\ d^{-1} \pm 0.01$) was slightly but significantly lower than that ($0.87\ d^{-1} \pm 0.07$, Table 1) recorded in heterotrophic flask cultures (ANOVA $F = 12.35$, d.f 1,7, $p < 0.001$).

### 3.3. *Effect of trophic mode on dissolved gas concentration, photosynthesis and respiration*

In order to investigate the existence and growth effect of mixotrophic synergistic gas exchange; the DIC concentration,

**Table 2 – Chlorophyll analysis of *M. inermum* under different trophic conditions.**

| Culture conditions | Total chlorophyll content (% dry cell weight) | | |
|---|---|---|---|
| | Exponential[a] | Initial Stationary[b] | End Stationary[c] |
| Autotrophic | 2.53 ± 0.17[A] | 2.44 ± 0.15[A] | 2.29 ± 0.06[AB] |
| Mixotrophic (Sodium acetate) | 1.89 ± 0.01[CD] | 1.65 ± 0.07[C] | 2.03 ± 0.04[BD] |
| Heterotrophic (Sodium acetate) | 0.73 ± 0.01[E] | 0.78 ± 0.01[E] | 0.77 ± 0.06[E] |
| Mixotrophic (Glucose) | 0.28 ± 0.02[F] | ND | ND |
| Heterotrophic (Glucose) | 0.064 ± 0.01[G] | ND | ND |

Values denoted by a different upper case letter at each data point differ significantly at $p < 0.05$ by log transform. ND represents data not determined.

[a] Expontential phase – samples taken after 2 days (mixotrophic and heterotrophic glucose), 3 days (heterotrophic acetate) and (21 days autotrophic).

[b] Initial stationary phase – samples taken after 4 days (mixotrophic), 8 days (heterotrophic), 28 days (autotrophic). By this point mixotrophic and heterotrophic growth was limited by availability of acetate but autotrophic cultures were still growing (and unlikely to be nutrient limited).

[c] End stationary phase – samples taken after 49 days (mixotrophy and heterotrophy), 70 days (autotrophy). By this point autotrophic cultures were still not at a stationary phase.

DO concentration, photosynthetic rate, and respiration rate of cultures were analysed daily for each trophic condition over a 7 day period. In parallel, DO and DIC measurements were taken of non-inoculated sterile medium over the course of the experiment to give an abiotic air-equilibrated control. The control was subject to the same experimental conditions as autotrophic cultures. In order to avoid depleting the culture volume, two parallel fed batch experiments were carried out: experiment a, measuring DIC, and experiment b, measuring DO, photosynthetic rate and respiration rate. As shown in Fig. S2 in the supplementary material, the growth curves of cultures in each experiment were similar, enabling a direct comparison between results of the two experiments.

The DIC concentration in autotrophic cultures dropped significantly below the air-equilibrated control culture within 24 h (0.008 mM C ± 0.0001). Beyond 24 h the DIC remained relatively stable (Fig. 3). This trend was matched by an initial small but significant accumulation of DO concentration above the air equilibrated control after the first 24 h. Beyond this point there was no significant difference from the control (Fig. 4). Oxygen evolution measurements show that after 24 h (the first measurement point) rates of autotrophic photosynthesis were low and did not significantly differ throughout the experiment (Fig. 5).

The DIC concentration in heterotrophic cultures increased steadily over time as cell density increased, reaching 0.74 mM C ± 0.01 after 7 days, compared to 0.04 mM C ± 0.01 in the air-equilibrated control (Fig. 3). Similarly, the DO in heterotrophic cultures steadily decreased over time reaching 0.06 mM $O_2$ ± 0.008, compared to 0.27 mM ± 0.001 in the air-equilibrated control (Fig. 4). The rates of photosynthesis and respiration (μM $O_2$ per mg $DCW^{-1}$ $min^{1}$) over the growth period are shown in Fig. 5. Over a 48 h period respiration significantly increased in heterotrophic cultures during a period of exponential growth. Beyond 48 h there was a weak decline in respiration rate, but only significant at 144 h (Fig. 5). Despite having a significantly higher DIC, the heterotrophic photosynthesis rate did not significantly differ from autotrophic cultures. After 48 h there was a statistically significant (60%, Fig. 5) increase in the rate of photosynthesis in

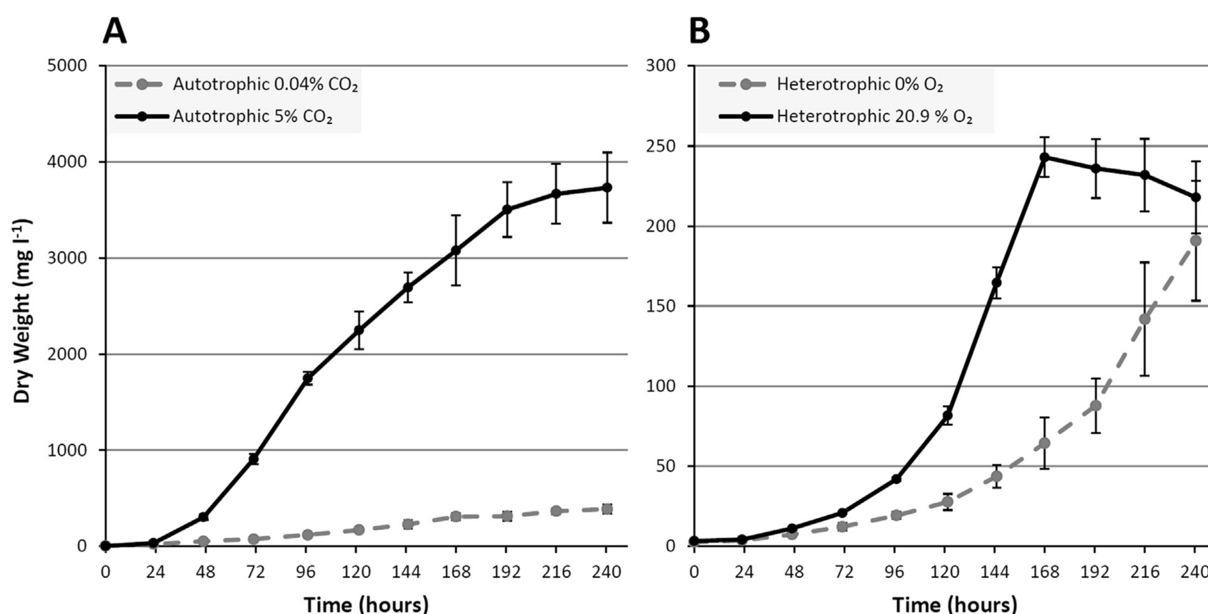


**Fig. 2 – A. Growth curve of autotrophic *M. inermum* under 0.04% (atmospheric air) and 5% $CO_2$ (air balanced) aeration. B. Growth curve of heterotrophic *M. inermum* under 0% $O_2$ (100% $N_2$) and 21% $O_2$ (atmospheric air) aeration. Cultures were grown in miniature bioreactors (100 ml volume).**

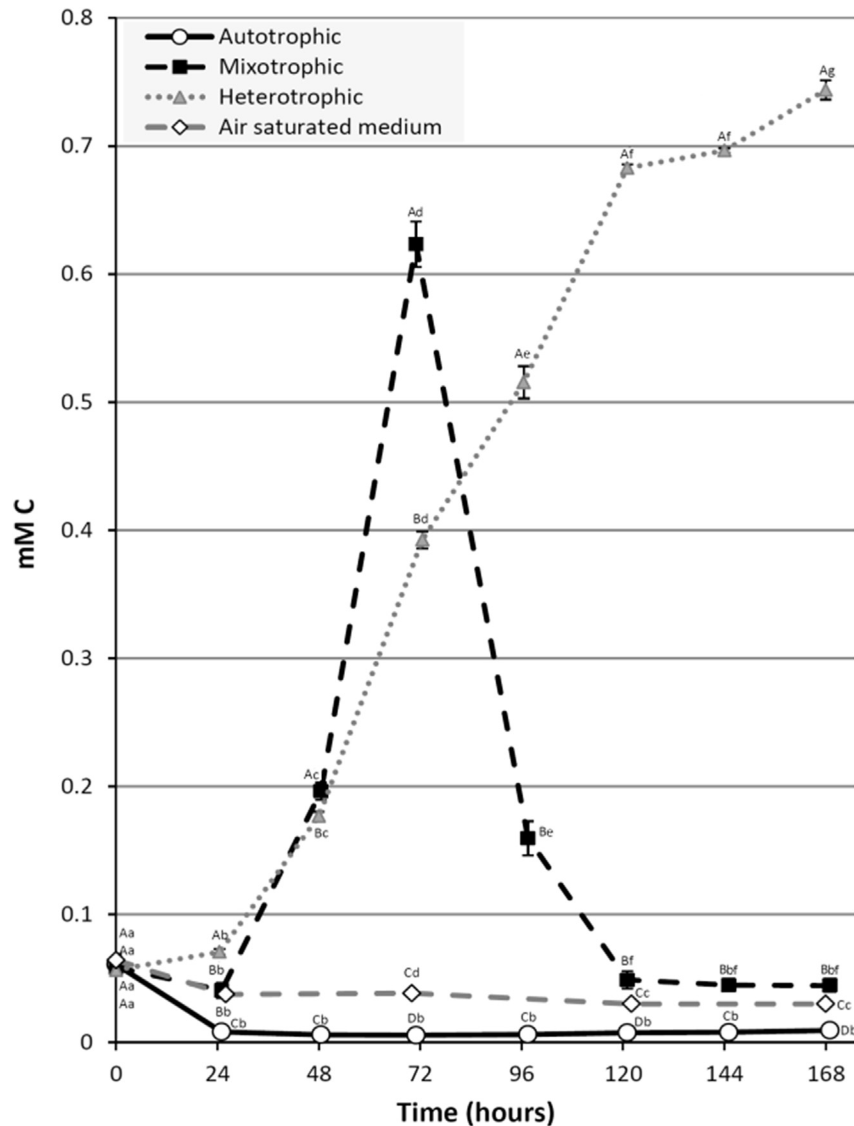


**Fig. 3 – DIC concentration (mM C) of *M.inermum* cultures under different trophic conditions. Values denoted by a different letter at each data point differ significantly at $p < 0.05$. Uppercase letters correspond to significance between treatments within time points, analysis was performed by one-way ANOVA followed by Tukey analysis, Lowercase letters correspond to significance between time points within treatments, analysis was performed by repeated measures nested ANOVA and Tukey analysis. Data was log transformed for intra-treatment statistical analysis.**

heterotrophic cultures, this remained relatively stable throughout the experiment despite continually rising DIC.

As demonstrated in Figs. 3 and 4, mixotrophic cultures showed significant variability in both dissolved gas concentrations throughout the experiment. After 48 h both the DIC and DO concentration in mixotrophic cultures increased significantly (Figs. 3 and 4). DIC concentration in mixotrophic cultures was 4.9 and 32.7 times higher than the air-equilibrated control and autotrophic cultures respectively. Similarly, the DO concentration in mixotrophic cultures was 1.6 and 2.4 times higher than the air-equilibrated control and heterotrophic cultures respectively. The substantial increase in dissolved gases was synchronous with significantly enhanced rates of both photosynthesis and respiration (Fig. 5). At 48 h the average rate of photosynthesis per mg DCW was 3.9 times higher than autotrophic cultures. Similarly, average respiration rates were 2.7 times higher than heterotrophic cultures.

Over the next 24 h both DO and DIC concentrations of the mixotrophic cultures increased further (Figs. 3 and 4). Despite the increase in DIC concentration, between 48 and 72 h respiration was reduced by a factor of 5.7 (Fig. 5). Photosynthetic activity also significantly decreased, although still remaining 2.27 times higher than autotrophic cultures (Fig. 5). Beyond 72 h there was significant decline in both DIC and DO concentration to levels just above saturation (Figs. 3 and 4).

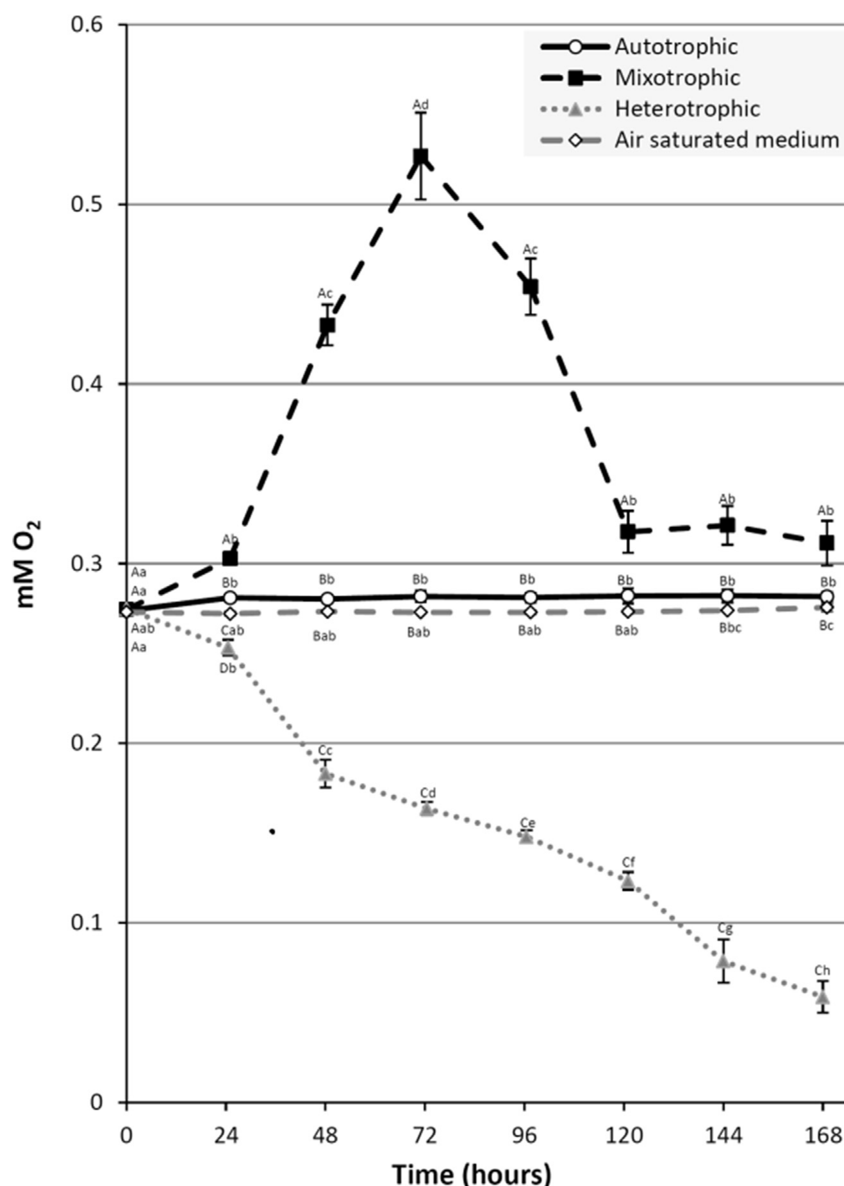


**Fig. 4 – DO concentration (mM $O_2$) of *M. inermum* cultures under different trophic conditions. Values denoted by a different letter at each data point differ significantly at $p < 0.05$. Uppercase letters correspond to significance between treatments within time points, analysis was performed by one-way ANOVA followed by Tukey analysis, Lowercase letters correspond to significance between time points within treatments, analysis was performed by repeated measures nested ANOVA and Tukey analysis. Data was log transformed for intra-treatment statistical analysis.**

The decline in DIC and DO was mirrored by gradually decreasing rates of respiration and photosynthesis (Fig. 5).

## 4. Discussion

Two major constraints to the commercialisation of microalgae biofuels are high production costs and availability of concentrated $CO_2$ [11]. Mixotrophic cultivation through the supplementation of waste organic carbon sources is a promising strategy to significantly increase productivity [26]. Results from previous studies demonstrated that under non-aerated conditions the specific growth rate of mixotrophic cultures is higher than the sum of autotrophic and heterotrophic growth (Table 1)[15]. This additional growth is not apparent when autotrophic and heterotrophic cultures are aerated. In this study, a locally isolated strain of chlorella like green algae *M. inermum* showed a similar pattern of growth, mixotrophic cultures supplemented with acetate or glucose had a specific growth rate 1.74 and 1.37 times the sum of autotrophic and heterotrophic growth respectively under non-aerated conditions (Fig. 1, Table 1).

**Fig. 5 – Photosynthesis and respiration rates (μM $O_2$ mg $DCW^{-1}$ $min^{-1}$) of *M.inermum* under autotrophic, heterotrophic (sodium acetate) and mixotrophic (sodium acetate) conditions. Values denoted by a different letter at each data point differ significantly at $p < 0.05$. Uppercase letters correspond to significance between treatments within time points, analysis was performed by one-way ANOVA followed by Tukey analysis, Lowercase letters correspond to significance between time points within treatments, analysis was performed by repeated measures nested ANOVA and Tukey analysis. Mixotrophic intra-treatment data was log transformed to meet the assumptions of statistically analysis.**

The faster growth rates found in glucose supplemented cultures are consistent with previous studies and this preference can be attributed to the higher energy content (2.8 kJ $mol^{-1}$) compared to acetate (0.8 kJ $mol^{-1}$) [27]. Chlorophyll analysis of the cultures under different trophic conditions revealed a significant reduction in chlorophyll content in both the mixotrophic and heterotrophic glucose supplemented cultures. Stadnichuk et al. [28] reported that, amongst other physiological changes, glucose inhibits the biosynthesis of chlorophyll *a* in the red alga *Galdieria sulfuraria* by partially blocking the transformation of a precursor molecule coproporphyrin III. This synthesis inhibition is reduced under mixotrophic conditions as light stimulates the production of coproporphyrin III [28]. While many studies have reported reduced chlorophyll content in glucose supplemented cultures, the degree of chlorophyll synthesis reduction varies substantially between species and strains [16,26,28]. In *Micractinium inermum* the large reduction in chlorophyll content in glucose supplemented cultures is likely to have reduced photosynthetic efficiency, which provides a reasonable explanation for the reduced specific growth rate ratio compared to acetate supplemented growth. This point highlights the large variability in physiological changes caused by supplementation of different organic carbon sources.

It was hypothesised that the enhanced growth rate under mixotrophic conditions was due to synchronous activity of the respiratory and photosynthetic metabolic components. These components could act synergistically, providing a source of endogenic $CO_2$ and $O_2$ which in turn reduces growth limitation and thus enhances growth. Firstly, in order to confirm that the growth of the, previously uncharacterised, *M. inermum* was limited by the availability of $CO_2$ and $O_2$ respectively, cultures were grown under aerated conditions (Fig. 2A and B). Significant increases in the growth rate of autotrophic cultures under both air and 5% $CO_2$ (balance air) aeration clearly demonstrates that availability of DIC is a key

limiting factor for growth in autotrophic *M. inermum culture* (Fig. 2A). This phenomenon is well understood and is consistent with previous work investigating the effect of DIC concentration on growth of other algae species [29,30].

In contrast to autotrophic growth, previous studies have shown that in heterotrophic microalgae cultivation oxygen supply is a key limiting factor [27,31] as would certainly be expected. In this study air aerated cultures had a significantly higher specific growth rate when compared to $N_2$ sparged cultures. The peak and subsequent decline in biomass concentration of air-aerated cultures after 168 h of growth was likely to be due to acetate depletion (Fig. 2B). A similar pattern of decline occurred in both acetate and glucose supplemented cultures in the flask experiment (Fig. 1). Surprisingly, although the $N_2$ sparged cultures had slower growth than the air aerated treatment, the cultures still reached a relatively high biomass concentration (Fig. 2B). Dissolved oxygen measurements of the aerated cultures (Fig. S3 in the supplementary data) indicated that the slow flow rate of the $N_2$ aeration did not lead to oxygen stripping, but followed a pattern similar to that found in flask cultures (Fig. 4). The limited oxygen stripping in the $N_2$ aerated control culture was mostly likely due to a combination of low flow rate (10 $cm^3$ $min^{-1}$) and the intermittent high flux of air used to resuspend cells accumulated in the dead zones of the reactor. Rather than comprising the conditions of the control this slow oxygen limitation mimicked the growth conditions of the flask culture more closely than a control with immediate oxygen stripping. Despite this similarity, both aerated treatments had a lower specific growth rate than acetate supplemented non-aerated flask cultures. Given the high ambient concentrations of dissolved oxygen, the growth during the early log phase would not be expected to be significantly elevated or depressed due to the treatment conditions. Both aerated cultures had a more pronounced lag period in the first 24 h of growth than the equivalent flask culture, it is possible this difference may have been due to different inoculum and culture conditions used (constantly mixed air loop reactors rather than flasks). Consequently it is difficult to draw strong conclusions based on data from the early log phase of growth (i.e specific growth rate). Nethertheless, enhanced productivity during later stages of growth in the air-aerated cultures indicates that oxygen availability is a limiting factor in heterotrophic growth of *M. inermum* but to a greater degree at higher cell densities. Comparatively, $O_2$ limitation in heterotrophs is small relative to $CO_2$ limitation in autotrophic cultivation, which is expected due to the ambient concentration of gases. Therefore, the effect of any mixotrophic gas exchange is likely to enhance photosynthetic activity to a greater degree than respiration, especially at low cell densities.

Finally, in order to test the hypothesis of synergistic gas exchange in mixotrophic cultures the DIC concentration, DO concentration and rates of photosynthesis and respiration were measured in autotrophic, mixotrophic and heterotrophic cultures. It was expected that during the growth period the DIC concentration would increase in heterotrophic cultures but decline in autotrophic cultures. The reverse was expected for the DO concentration. This pattern would result in increasing limitation on photosynthesis and respiration for autotrophic and heterotrophic cultures respectively. In contrast, within mixotrophic cultures it was expected that the activity of both metabolic pathways would lead to relatively more stable concentrations of both DIC and DO.

The results show that autotrophic and heterotrophic cultures largely followed the predicted trend. Autotrophic cultures quickly became depleted in DIC and remained at a stable low level (Fig. 3). It is likely that the photosynthetic consumption of DIC by the autotrophic algae was met by mass transfer of $CO_2$ into the culture (once below equilibrium levels). The depletion in DIC was matched with no significant accumulation of DO and a low level of photosynthesis (Figs. 4 and 5). These data support the conclusions of the previous section, that photosynthesis in non-aerated autotrophic *M.inermum* cultures is significantly limited by the availability of DIC.

As predicted in heterotrophic cultures the metabolism of acetate led to a significant accumulation of DIC and depletion of DO (Figs. 3 and 4). After an initial significant increase in respiration, there was slight decline (although only significant at 144 h) in the rate of respiration (Fig. 5). Whilst the decline does support the hypothesis of oxygen limitation, given the disparately pronounced depletion of dissolved oxygen, strong conclusions cannot be made from this data. The limited photosynthetic rate in heterotrophic cultures despite a high concentration of DIC is likely to be due to a combination of low photosynthetic efficiency due to low level of cellular chlorophyll and the short period of photosynthesis induction (cultures only exposed to light for 6 min during oxygen evolution measurements) [32,33].

In contrast to the expected stability, mixotrophic cultures showed the most variability in DIC and DO concentration. This variability reflects the rapid growth of mixotrophic cultures during a short log phase, followed by depletion of acetate and a gradual acclimation to autotrophic conditions. This trend is supported by the increased chlorophyll content during the stationary phase of growth (Table 2). The substantial increases in dissolved gases during log growth (first 48 h) were synchronous with significantly enhanced rates of both photosynthesis and respiration. This correlation strongly indicates that the mixotrophic endogenic production of $CO_2$ and $O_2$ significantly stimulated metabolic activity.

In contrast to the weak trend found in heterotrophic cultures, these data indicate that respiration is significantly increased under conditions of high oxygen concentrations. This trend is supported by the significantly higher productivity found in the air-aerated heterotrophic relative to the control and non-aerated flask culture (Fig. 2B). Previous studies on species within the closely related *Chlorella* genus, have shown that DCMU addition (an inhibitor of electron transport from PSII to PSI) to mixotrophic cultures reduces growth and respiration rates to heterotrophic values [13,15]. However, because of the difficulty of separating rates of active photosynthesis and respiration under illumination in oxygen electrode experiments, these studies do not give any indication on the impact of photosynthesis or illumination on the efficiency of acetate assimilation and metabolism. Consequently it is possible that the augmented respiration rates in mixotrophic cultures could be influenced by light rather than or in addition to oxygen concentration. Further research is needed before strong conclusions about the effect of illumination and

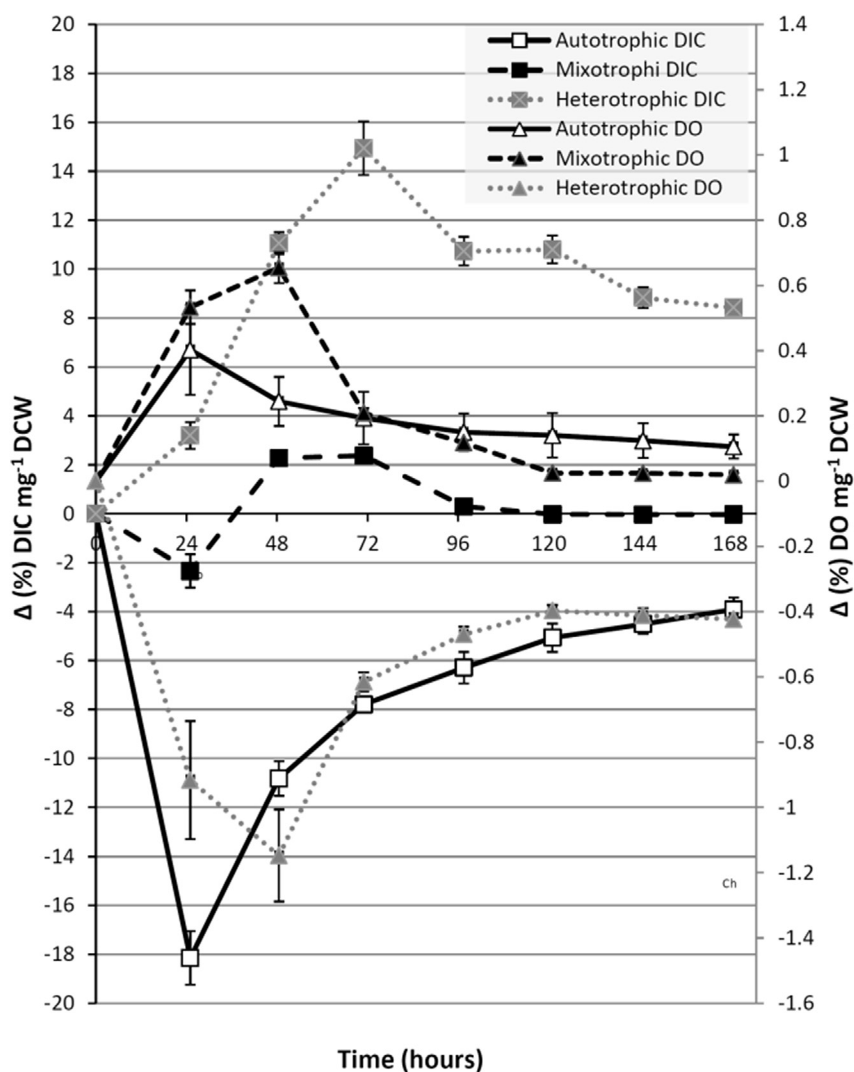


**Fig. 6 – Change (%) per mg DCW in DIC and DO concentration (from original values) of *M.inermum* cultures under different trophic conditions. Bars represent standard deviation.**

oxygen availability on the respiration, acetate uptake and growth of *M.inermum* can be made.

The synchronous increase of both dissolved gases appears paradoxical as one would expect either a dominance of one metabolic pathway causing accumulation of inorganic carbon and depletion of oxygen or vice versa. However as demonstrated in Fig. 6, showing the % change in both DIC and DO per unit of biomass, the accumulation of both gases can be explained by shifting dominance of the contrasting metabolic pathways during the initial growth period. Over the first 24 h accumulation of DO and decline in DIC concentration indicates net autotrophic growth (Fig. 6). Beyond this point, the DIC, DO and oxygen evolution measurements indicate rapidly increasing rates of respiration, accumulation of DIC and reduction in the rate of oxygen accumulation per unit of biomass (Figs. 5 and 6). This initial photosynthetic dominance suggests a lag phase in the expression of enzymes involved in the metabolism of acetate. Such a phenomenon has been reported in *Chlorella sorokiniana*, where increasing acetate concentration led to a longer lag period of growth [34]. This phenomenon has been reproduced in *M. inermum* (Fig. S4, in the supplementary data). It is possible that this pattern reflects an increase in light limitation and a shift to a heterotrophic pathway, however given the relatively low cell density during the second day of growth this explanation is unlikely (Fig. S2). Although the rapid production of $CO_2$ fuelled an increase in the rate of photosynthesis, allowing the mixotrophic cultures to remain net autotrophic (Fig. 5), it is possible that $CO_2$ production exceeded the rate at which it could be sequestered via the Calvin–Benson cycle, leading to DIC accumulation and diffusion into the media [35]. Alternatively, even if the rate of sequestration met the rate of production, high levels of $CO_2$ and $O_2$ production at different sites within the cell (mitochondria and chloroplast) may have led to a diffusive loss from the cell into the media. Again the dynamics of this gas

exchange deserves more in depth research before any strong conclusions can be made.

At 72 h both DO and DIC concentrations of the mixotrophic culture increased further (Figs. 3 and 4). Despite the increase in DIC, between 48 and 72 h respiration was reduced by a factor of 5.7 (Fig. 5). This disparity can be explained by the fact that the oxygen evolution measurements represent a snap shot at that time, rather than the more cumulative effect represented by dissolved gas measurements. The decline in respiration was synchronous with the end of exponential growth and was followed by a sudden drop in both DIC and DO within 24 h (Figs. 3 and 4), indicating that acetate was exhausted from the medium. Given the significantly elevated respiratory activity at 48 h in high DIC, it is unlikely that high DIC concentration limited heterotrophic respiration as previously reported in *Chlorella protothecoides and Nannochloropsis salina* [36]. Despite the peak in DIC concentration, at 72 h photosynthetic activity also significantly decreased, although still remaining 2.27 times higher than autotrophic cultures (Fig. 5). Given the four fold increase in cell density, it is probable that, at this point, self-shading induced light limitation prevented maximum rates of photosynthesis [37].

Although the pattern of dissolved gas concentrations did not follow the predicted stability in mixotrophic cultures, if measurements given in Figs. 3 and 4 are standardised to a per mg DCW basis, the percentage change in DIC in mixotrophic cultures after 48 h (from initial value) were nearly 5 times less than the change observed in heterotrophic cultures (Fig. 6). Correspondingly, at 24 h the percentage change in DO in autotrophic cultures were only 25% lower than mixotrophs, despite autotrophs being significantly limited by DIC availability. The apparent ability of mixotrophic metabolic activity to stabilise DO concentrations relative to autotrophic growth may provide important additional benefits in reducing photorespiration and photoinhibition induced by oxygen accumulation [38,39].

## 5. Conclusion

In summary, acetate-fed mixotrophic *Micractinium inermum* cultures were found to have a specific growth rate 1.74 times the sum of autotrophic and heterotrophic growth under non-aerated condition. Changes in DIC, DO and rates of photosynthesis and respiration were tracked in autotrophic, mixotrophic and heterotrophic *M. inermum* cultures over a 7 day growth period. The significantly enhanced metabolic activity, augmented growth rates and comparative stability of dissolved gas concentrations support the hypothesis of synergistic gas exchange between photosynthetic and respiratory metabolic pathways within mixotrophic cultures. The data provide strong evidence that endogenic production of $CO_2$ significantly reduces inorganic carbon limitation of photosynthetic activity. In addition, the results provide some, albeit weaker, evidence for increased respiratory activity through photosynthetic production of $O_2$. Further work is needed to elucidate whether enhanced mixotrophic respiration is due to reduced oxygen limitation or greater efficiency of acetate metabolism under illuminated conditions in *M. inermum*. Endogenic production of $CO_2$ in particular could remove or reduce the costs of aeration in large scale production systems and importantly reduce reliance on limited sources of concentrated $CO_2$. In addition, the consumption of photosynthetically derived $O_2$ by enhanced respiration in mixotrophic cultures could reduce oxygen stripping costs associated with autotrophic microalgae production systems. This work has also highlighted that the benefits of mixotrophy are very dependent on the species and organic carbon compound utilised. Sustainable mixotrophic growth requires the use of waste organics, consequently further research is required to investigate whether the benefits of synergistic gas exchange can be realised with other organic carbon sources typically found in waste streams suitable for algal growth.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biombioe.2015.04.023.

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