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RESEARCH

Organic carbon utilisation by the filamentous alga *Tribonema*

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Abstract
Filamentous algae have potential application to wastewater treatment, in particular for efficient recovery of nutrients into biomass. However, supplying inorganic carbon is a major limiting factor. The utilisation of organic carbon present in wastewater may reduce the constraints in carbon supply, however there is little knowledge of mixotrophic growth amongst filamentous algae. This study investigated the utilisation of organic carbon sources relevant to wastewater by the filamentous xanthophyte alga *Tribonema*. Algae growth was compared in the absence of organic carbon (autotrophic) and in presence of 0.2 g-C L^{-1} glucose, ethanol or acetate under mixotrophic (presence of organic carbon and light) or heterotrophic (presence of organic carbon and absence of light) conditions. To investigate direct utilisation of organic carbon and indirect utilisation via bacterial CO_2-genesis, cultivation was performed under both axenic and non-axenic conditions. *Tribonema* was found to directly utilise glucose, which increased mixotrophic productivity and maintained growth under heterotrophic conditions. In contrast, acetate was only indirectly utilised mixotrophically in the presence of bacteria, whereas ethanol was not utilised under any conditions. The underlying mechanisms of glucose utilisation by *Tribonema* were also investigated by analysing its photosynthetic rate and respiration rate under glucose concentrations ranging from 0 – 100 g L^{-1}. Based on the results, enhancements to metabolic pathways and reduced CO_2 requirements provided by glucose utilisation were proposed. Despite the positive results with respect to glucose utilisation, out competition for this resource by bacteria suggest that *Tribonema* is more suitable for treatment of wastewater with low organic carbon concentrations, such as secondary-treated wastewater effluent.

Keywords Algal mixotrophy · Xanthophyceae · Wastewater treatment · Algal cultivation · Algae-bacteria interaction · Glucose metabolism

Introduction

The use of algal processes in wastewater treatment is energy-efficient, while also generating useful end products by assimilation of carbon and nutrients (Chen et al. 2015; Liu et al. 2017; Mohd Udaiyappan et al. 2017). An effective algal system, either for nutrient removal or biomass cultivation, requires the areal productivity to be maximised. There are various factors that can affect the productivity of algae, including species selection, carbon supply, nutrient availability, and algae-bacteria interactions (Liu et al. 2020). For large-scale algae cultivation, carbon availability is usually a major limiting factor on productivity unless external inorganic carbon supplementation is provided, for example by gas sparging. This is due to the intense photosynthetic requirements, slow water exchange and gas diffusion, and the relatively low proportion of free CO_2 at high pH (Denny 1990; Menéndez et al. 2001; Mata et al. 2007; Cole et al. 2014).

While cultivation of filamentous algae has advantages in wastewater treatment compared to microalgae such as the ease of biomass retention and harvesting (Liu et al. 2020), carbon provision in these cultures can be more difficult. Unlike unicellular microalgae that can be fully suspended in water, the long strands of the filamentous algae form macroscopic structures such as floating mats. This physical behaviour provides extra advantages such as a higher resistance to predation and better access to surface light, but they

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are highly susceptible to mixing and shear during cultivation, making aeration problematic. Further, the presence of stagnant surface mats means gas exchange between air and water can be limited, which exacerbates the inorganic carbon supply issue for filamentous algae cultivation.

One possible solution is to provide organic carbon sources that are not dependent on air-water gas exchange. Wastewater usually contains a complex mixture of organic carbon substances, which can be categorised based on size, solubility, biodegradability and hydrophobicity (Tran et al. 2015). The detailed composition is usually highly dynamic and complicated. However, the biodegradable organic carbon present in wastewater is usually converted to smaller molecules under biological activities during wastewater treatment processes, which can be suitable for further treatment using algal technologies (Law et al. 2013; Chan et al. 2022). Domestic wastewater already contains high levels of organic carbon (typically 109 – 328 mg L^{-1} (Medcalf & Eddy Inc et al 2014)) that can potentially be utilised by mixotrophic algae in addition to inorganic carbon sources. In addition, organic carbon sources, for example glucose, can be deliberately added to a wastewater stream as substrates to enhance the efficiency of treatment processes (Leal et al. 2016; Li et al. 2021). Mixotrophic growth of algae is a commonly studied phenomenon, with numerous studies showing enhancement of algal productivity in the presence of organic carbon molecules such as glucose, sucrose, acetate and glycerol (Bhatnagar et al. 2011; Moon et al. 2013; Poddar et al. 2018; Patel et al. 2019). This enhancement can be due to both the direct utilisation of these molecules by algae and the indirect utilisation of CO_2 as a metabolic end-product from the surrounding bacteria. Although it is known that the contribution of bacterial CO_2 to algal growth can be significant, studies uncoupling the effect of algae and bacteria are also required to better understand the potential balanced between direct and indirect utilisation by algae in order to design and optimise large-scale processes. While many studies have investigated mixotrophy in microalgae, only a few studies have looked at this aspect of carbon utilisation for freshwater filamentous algae. These studies demonstrated the ability of *Tribonema* and *Spirogyra* to utilise various forms of organic carbon sources, including glucose and acetate, for enhanced mixotrophic growth (Wang et al. 2017, 2023; Amiri and Ahmadi 2020). However, there was no elimination of heterotrophic bacteria in this study, making it unclear whether the uptake was due to direct utilisation of organic carbon, or of CO_2 from bacterial metabolism. The ground-breaking work by Belcher and Fogg (1958) specifically identified chemo-organotrophic growth of *Tribonema* using organic carbon under bacteria-free conditions. Glucose promoted the strongest growth, followed by acetate and citrate, with sucrose utilised heterotrophically following adaptation. Utilisation of glucose by *Tribomena* has more recently been confirmed by others, showing enhanced productivity compared to photoautotrophic conditions (Wang et al. 2017; Přibyl and Cepák 2019; Zhou et al. 2019). However, a direct comparison in the presence and absence of bacteria has not been presented, and the underlying mechanisms for organic carbon utilisation and algae-bacteria interaction has not been discussed, leaving a critical research gap especially for wastewater treatment applications.

This study aimed to further understand the ability of *Tribonema* to utilise organic carbon for mixotrophic and heterotrophic growth. The genus *Tribonema* is endemic in the wastewater treatment lagoon system in Melbourne, Australia, and was selected among other filamentous algae due to previously demonstrated potential for domestic wastewater treatment (Cheng et al. 2020; Liu et al. 2023). Experiments were conducted under axenic and non-axenic conditions to disentangle algal organic carbon utilisation from the algal-bacterial relationship during mixotrophic and heterotrophic growth. The possible metabolic pathways for organic carbon utilisation are discussed, based on the experimental findings on the algal productivity, photosynthetic rate and respiration rate.

Materials and methods

Filamentous algae

A previously studied (Liu et al. 2023) filamentous alga from the genus *Tribonema* was used in this study. Prior to the experiments, stock cultures of *Tribonema* were cultivated for 7 days under autotrophic conditions in 25 cm^2 cell culture flasks (Corning) using MLA medium containing 28 mg-N L^{-1} $NaNO_3$ as nitrogen source (Liu et al. 2023), which was modified, as specified later. The stock cultures were then harvested by pouring the whole culture containing both the growth medium and algae through a stainless-steel strainer with a pore size of <700 µm. The filaments retained at the base of the strainer were then easily picked up with a tweezer, with the biomass sampled for FW:DW ratio measurement and inoculated for the experiments herein.

Experiments on the effect of organic carbon on filamentous algal growth

To investigate the effect of organic carbon on the productivity of *Tribonema*, growth experiments were performed autotrophically (absence of organic carbon under a day/night light cycle), mixotrophically (presence of organic carbon under a day/night light cycle), and heterotrophically (presence of organic carbon under complete darkness), under both axenic and non-axenic conditions.

For all experiments, MLA medium (Bolch and Blackburn 1996) was used; alone for the autotrophic condition, and with glucose, ethanol or acetate included as an organic carbon source for the mixotrophic and heterotrophic conditions. Considering the typical productivity of *Tribonema* cultivated in the lab and the corresponding amount of organic carbon utilisation anticipated (if any), a low initial concentration for the organic carbon (0.2 g-C L^{-1}) was selected for the experiments. The selected concentration was also representative of the typical organic carbon level in domestic wastewater, as identified in the Introduction section. To achieve 0.2 g-C L^{-1}, 0.5 g $L^{-1}$of anhydrous D-glucose, 0.5 mL of 100% ethanol, or 0.475 mL of pure acetic acid were added to each litre of the MLA medium. For the acetate-containing medium, addition of 1M NaOH was required to raise the pH to 8.3. The media were then filtered with a vacuum filter using 0.22 µm filter paper (Millipore), and for the axenic cultures, 200 mg L^{-1} ampicillin (sodium salt) was added to the medium. Samples of the medium were taken after preparation, syringe filtered using 0.22 µm PES membrane (Millex) and stored at -20 °C until further characterisation.

For autotrophic and mixotrophic growth experiments, approximately 2.5 mg fresh weight (FW) of biomass was inoculated into 25 cm^2 cell culture flasks containing 20 mL of the modified MLA medium. The cultures had an illuminated growth area of 29.5 cm^2. A 14h/10h light/dark cycle was used for the experiments with a light intensity of 100 µmol photons $m^{-2}s^{-1}$ and temperature of 15 °C. All samples were cultivated without mixing or aeration in a 600 L reach-in growth chamber (BI-RIC-600, Biora, Australia) to maintain constant light and temperature conditions. For heterotrophic growth, the experiments were performed in an identical way as the other two conditions, except that the culture flasks were completely covered with aluminium foil to prevent access to light. Triplicate experiments were performed under the three different growth conditions, using three different organic carbon sources either axenically or non-axenically.

All samples were harvested after a 7-day cultivation period. A sample of the medium was first extracted from each culture flask with a pipette, then syringe-filtered and stored at -20 °C until further characterization. The remaining content in each culture flask was vacuum filtered through 0.22 µm filter paper. The algal biomass retained on the filter paper was then rinsed with deionised water for 10 s, recovered using a fine tweezer and placed in an oven at 60 °C for at least 24 h prior to measuring its dry weight (DW). The amount of biomass retained was then determined, and the areal productivity of algae calculated as:

$$\text{Areal Productivity } \left(\text{gDW m}^{-2}\text{ day}^{-1}\right) = \left(\text{M}_\text{f} - \text{M}_\text{i}\right)/(\text{A} \times \text{d}),$$

where M_f and M_i are the final and initial dry weight of the algae (g), A is the growth area (m^2), and d is the duration of growth (day).

The amount of carbon assimilated into the biomass was also calculated as follows:

$$\text{Amount of carbon assimilated in biomass (mg)} = \left(\text{M}_\text{f} - \text{M}_\text{i}\right) \times \text{p}$$

where M_f and M_i are the final and initial dry weight of the algae (mg), and p is the carbon content in the dry biomass which was assumed to be 45% based on typical values in the literature (Lawton et al. 2013).

It is worth noting that only algal biomass recoverable using a tweezer included in DW determinations and areal productivity calculations. Any weak, unhealthy filaments that could not be recovered were qualitatively identified as non-recoverable biomass and are generally non-viable in the long term.

The level of organic carbon in the medium before and after the experiment was then measured. Glucose, ethanol and acetate concentrations were measured using a Megazyme Sucrose/D-Fructose/D-Glucose assay kit, ethanol assay kit and acetic acid assay kit (AK analyser format) respectively according to the manufacturer instructions. Detailed calculations on algal growth, organic carbon consumption and overall carbon mass balance are presented in Supplementary S3.

Experiments on the effect of glucose on algal photosynthesis and respiration

To further investigate the effect of glucose on algal photosynthesis and respiration, experiments were performed to measure the photosynthetic rate and respiration rate based on the change in dissolved oxygen (DO) level in a closed system under different light intensities and glucose concentrations. The experimental procedure was adapted from a previous study using the same apparatus (Pitawala et al. 2023). Briefly, the apparatus was divided into three zones that were fully closed and separated using baffles with fine pores in between. This allowed mixing of the medium across the whole apparatus and simultaneous DO measurement, while eliminating shear to the algal filaments in the major compartment. The total volume of liquid in the apparatus was 255 mL when filled.

Prior to the experiments, stock cultures of *Tribonema* were cultivated for 7 days in 25 cm^2 cell culture flasks containing MLA medium and 200 mg L^{-1} ampicillin using a 14h/10h light/dark cycle with light intensity of 300 µmol photons $m^{-2}s^{-1}$ and temperature of 15 °C. To set up the experiment, the apparatus was almost filled with fresh MLA medium containing 200 mg L^{-1} ampicillin and different concentrations of glucose (0, 1, 10 or 100 g L^{-1}), then 50 – 100

mg FW of algal biomass was placed in the biomass chamber. More medium was then added slowly to completely fill the apparatus, with the lid then carefully closed to avoid entrapment of air bubbles. The screws were then hand-tightened to generate a closed system. The apparatus was then immediately placed on a magnetic stirring platform within the growth chamber. DO measurement was started and set to automatically record at 5 min intervals. Light was set to be on for the first 45 min, then turned off for the next 60 min, giving a total experimental period of 105 min. After the experiment, the biomass was harvested using a tweezer, rinsed with deionised water for 10 s, then dewatered using the method presented in Liu et al. (2023), and subsequently dried in an oven for at least 24 h prior to determine the DW.

A series of experiments was performed with different glucose concentrations 0, 1, 10 or 100 g L^{-1} in combination with different light intensities of 79, 337, 722 and 1093 µmol photons m^{-2} s^{-1}. The experiments were run in triplicate, with a blank experiment (no algal biomass) performed for each triplicate to determine the natural drift in DO level. For each sample and blank, the change in DO level was plotted against time to perform a linear regression for the subsequent calculation. To minimise data inaccuracy during transition, only data from the 15 - 45 min and 75 - 105 min were used for analysis of the light and dark periods respectively.

The photosynthetic rate and respiration rates of the algae were then calculated as:

$$\text{Photosynthetic rate } \left(\text{mg } O_2\ g^{-1}\ h^{-1}\right) = \left(S_s - S_b\right) \times f \times V/M_s$$
$$\text{Respiration rate } \left(\text{mg } O_2\ g^{-1}\ h^{-1}\right) = \left(S_s - S_b\right) \times f \times V/M_s$$

where S_s and S_b are the slopes obtained from linear regression of the sample and the blank respectively using data from the light period (for photosynthetic rate) or dark period (for respiration rate) (mg O_2 L^{-1} min^{-1}), f is the conversion factor of 60 for time (min h^{-1}), V is the volume of liquid in the apparatus (L), and M_s is the dry weight of the biomass (g).

Statistical analysis

For the experiments described in "Experiments on the effect of organic carbon on filamentous algal growth", two-sample *t*-tests were performed using Minitab to test statistical difference between some sample groups with a 95% confidence interval ($p < 0.05$). One-sample *t*-tests were also performed using Minitab to test statistical difference between some sample groups and 0 with a 95% confidence interval ($p < 0.05$).

For the experiments detailed in "Experiments on the effect of glucose on algal photosynthesis and respiration", two-sample *t*-tests were performed using Minitab to test statistical difference between the samples with a 95% confidence interval ($p < 0.05$). For each sample group the four data sets within the group were paired and compared with each other using two-sample *t*-tests.

Variation in the experimental data is presented as mean ± one standard deviation of the biological replicates.

Results

Effect of organic carbon on filamentous algae productivity

The areal productivity of *Tribonema* was compared when grown using different organic carbon sources under mixotrophic and heterotrophic conditions in axenic (Figure 1) and non-axenic (Figure 2) cultures.

Growth on glucose

With the addition of glucose, the productivity of *Tribonema* was significantly increased compared to the controls when grown mixotrophically under both axenic and non-axenic conditions, and heterotrophically under axenic conditions ($p < 0.05$). The productivity was similar regardless for axenic (0.304 ± 0.100 g m^{-2} day^{-1}) and non-axenic mixotrophic cultures (0.322 ± 0.069 g m^{-2} day^{-1}), both of which were higher than that under autotrophic conditions (0.147 ± 0.043 g m^{-2} day^{-1} for axenic, 0.143 ± 0.030 g m^{-2} day^{-1} for non-axenic). Under heterotrophic conditions, *Tribonema* was able to maintain some growth (0.077 ± 0.017 g m^{-2} day^{-1}) in the presence of glucose and antibiotics, although the productivity was significantly lower than that under autotrophic conditions ($p < 0.05$). In contrast, when grown heterotrophically and non-axenically with glucose, the *Tribonema* biomass was found to be unhealthy and friable and was not able to be harvested after the experiment.

Growth on acetate

In comparison to the increase in productivity when using glucose mixotrophically, the presence of acetate only increased the productivity of *Tribonema* under the mixotrophic and non-axenic condition (Figures 1 and 2). The productivity using acetate under the mixotrophic and axenic condition was only 0.099 ± 0.012 g m^{-2} day^{-1}), which was lower than under autotrophy (0.147 ± 0.043 g m^{-2} day^{-1}) although the difference was not statistically significant. In contrast, the productivity under the mixotrophic and non-axenic condition was 0.310 ± 0.011 g m^{-2} day^{-1} , which is significantly higher than autotrophy ($p < 0.05$) and very similar to the enhanced productivity using glucose. For heterotrophic growth, no increase in biomass was observed for both mixotrophic conditions, despite the filaments remaining

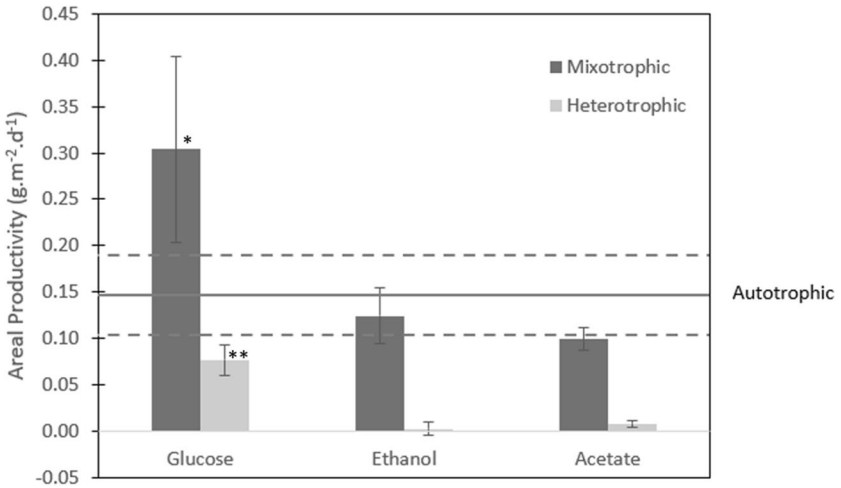

Fig. 1 Productivity of *Tribonema* using glucose, ethanol and acetate mixotrophically and heterotrophically under axenic conditions, compared to autotrophic growth without external organic carbon. Data for mixotrophic and heterotrophic growth are the average and standard deviation of three experimental replicates (n = 3). Data for autotrophic are the average (solid line) and standard deviation (dashed lines) of eight experimental replicates (n = 8). * indicates statistical significant difference for mixotrophic compared to autotrophic (p <0.05). ** indicates statistical significant difference for heterotrophic compared to 0 (p <0.05)

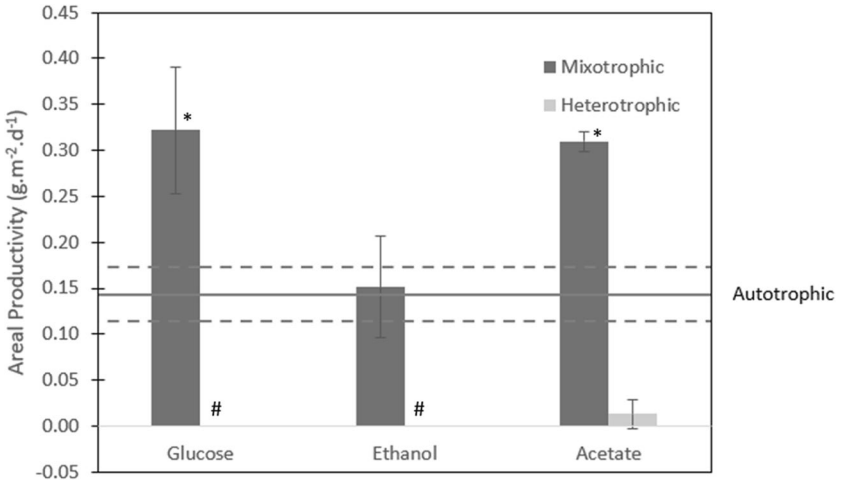

Fig. 2 Productivity of *Tribonema* using glucose, ethanol or acetate mixotrophically and heterotrophically under non-axenic conditions, compared to autotrophic growth without external organic carbon. Data for mixotrophic and heterotrophic growth are the average and standard deviation of three experimental replicates (n = 3). Data for autotrophic are the average (solid line) and standard deviation (dashed lines) of nine experimental replicates (n = 9). * indicates statistical significant difference for mixotrophic compared to autotrophic (p <0.05). # indicates no harvestable biomass was obtained for all replicates after the experimental period

green at end of the experimental period. The filaments in the axenic conditions remained relatively robust and easily harvestable, whereas the ones in the non-axenic conditions were relatively soft and easily broken, with one of the replicates being non-harvestable.

Growth on ethanol

The productivity of *Tribonema* did not seem to be significantly affected by the presence of ethanol. The areal productivity under mixotrophic conditions was 0.124 ± 0.030 g m^{-2} day^{-1} and 0.151 ± 0.055 g m^{-2} day^{-1} for axenic and non-axenic respectively, which were not statistically significant compared to autotrophy (0.147 ± 0.043 g m^{-2} day^{-1} for axenic, 0.143 ± 0.030 g m^{-2} day^{-1} for non-axenic). The productivity under heterotrophy was also not affected, with no increase in biomass for the axenic condition and biomass being non-harvestable for the non-axenic condition.

Glucose utilisation by *Tribonema*

Glucose consumption in axenic and non-axenic cultures

The productivity results clearly show enhanced growth of *Tribonema* in the presence of glucose. To better understand the carbon flux and the mechanisms of organic carbon utilisation, the extent of glucose consumption was determined. For non-axenic mixotrophic cultures, 85.6 ± 3.4% of the glucose was consumed during the experiment (Table 1), indicating active metabolism of glucose by bacteria and/or algae. Near complete glucose consumption (98.6 ± 1.2%) was also observed in the heterotrophic samples despite the algal biomass being non-harvestable. In comparison, in the axenic cultures, where bacteria were eliminated, the glucose present in the medium was only partially consumed during the experimental period under both mixotrophic and heterotrophic conditions (50.8 ± 22.9% and 34.6 ± 4.7%, respectively). Although not quantified, the non-axenic cultures were initially grown autotrophically and had very low levels of bacteria (confirmed by microscopic observations), with algal filaments representing the vast majority of the biomass in the system. The significant increase glucose consumption under non-axenic conditions containing only very low levels of bacteria, indicates much more efficient glucose utilisation by bacteria than by *Tribonema*.

Effect of glucose on photosynthetic and respiration rates

To better understand the role of glucose on the metabolism of *Tribonema*, the photosynthetic and respiration rates (Figure 3) were measured under different light intensities and glucose concentrations. The data shows that the photosynthetic rate increased as the glucose concentration increased. The changes were more prominent for the lower light intensity ranges (79 and 337 µmol photons m^{-2} s^{-1}) than at the highest light intensity. Interestingly, when light intensity increased, the photosynthetic rate only increased by a minimum amount for the groups with low glucose concentration (0 and 1 g L^{-1}), whereas no statistically significant changes were observed for the groups with higher glucose concentration (10 and 100 g L^{-1}). An increase in photosynthetic rate was also observed at the highest light intensity (1093 µmol photons m^{-2} s^{-1}) when glucose was absent (0 g L^{-1}), yet the reason for this remains uncertain.

The respiration rate of *Tribonema* was also measured under different light intensities and glucose concentrations. The respiration rate increased as a function of glucose concentration, with the trends being more prominent at lower light intensities. An increase in respiration rate as a function of light intensity can also be observed. However, the difference is not very pronounced, especially for the higher glucose concentration ranges.

Discussion

Acetate and ethanol are not utilised by *Tribonema*

Acetate

Results from the growth experiments using acetate showed the inability of *Tribonema* to utilise acetate without the presence of bacteria. In combination, the results (Figures 1 and 2) indicate that *Tribonema* growth was enhanced by increased CO_2 availability provided by bacterial metabolism of acetate, but that *Tribonema* could not directly utilise acetate. This was further confirmed by the observation that all acetate in the non-axenic samples was consumed after the cultivation period regardless of the growth of the algae. Similar to the glucose experiments, it can be inferred that the bacteria present in the growth medium quickly metabolised all available acetate, providing extra CO_2 for the carbon-limited environment and enhanced the productivity for the

Table 1 Biological carbon accumulation by *Tribonema* versus glucose-C consumption during the 7-day experimental period

Growth Conditions	Carbon accumulated in biomass (mg-C)	Glucose removed from medium (mg-C)	Percentage removal of glucose from medium	Glucose consumption vs biomass accumulation
Mixotrophic, axenic	2.82 ± 0.93	2.82 ± 1.27	50.8 ± 22.9%	accumulation = consumption
Mixotrophic, non-axenic	2.99 ± 0.64	4.75 ± 0.19	85.6 ± 3.4%	accumulation < consumption
Heterotrophic, axenic	0.71 ± 0.16	1.92 ± 0.26	34.6 ± 4.7%	accumulation < consumption
Heterotrophic, non-axenic	0*	4.87 ± 0.06	98.6 ± 1.2%	accumulation < consumption
Autotrophic, axenic	1.37 ± 0.40	0**	N/A	accumulation > consumption
Autotrophic, non-axenic	1.33 ± 0.27	0**	N/A	accumulation > consumption

The results were calculated based on change in harvested biomass DW and change in glucose concentration in the medium over 7-days. Data for mixotrophic and heterotrophic growth are the average and standard deviation of three replicates (n = 3). Data for autotrophic, axenic growth are the average and standard deviation of eight replicates (n = 8). Data for autotrophic, non-axenic growth are the average and standard deviation of nine replicates (n = 9). *No carbon accumulation recorded as the biomass was not harvestable at the end. ** No glucose was added to the samples

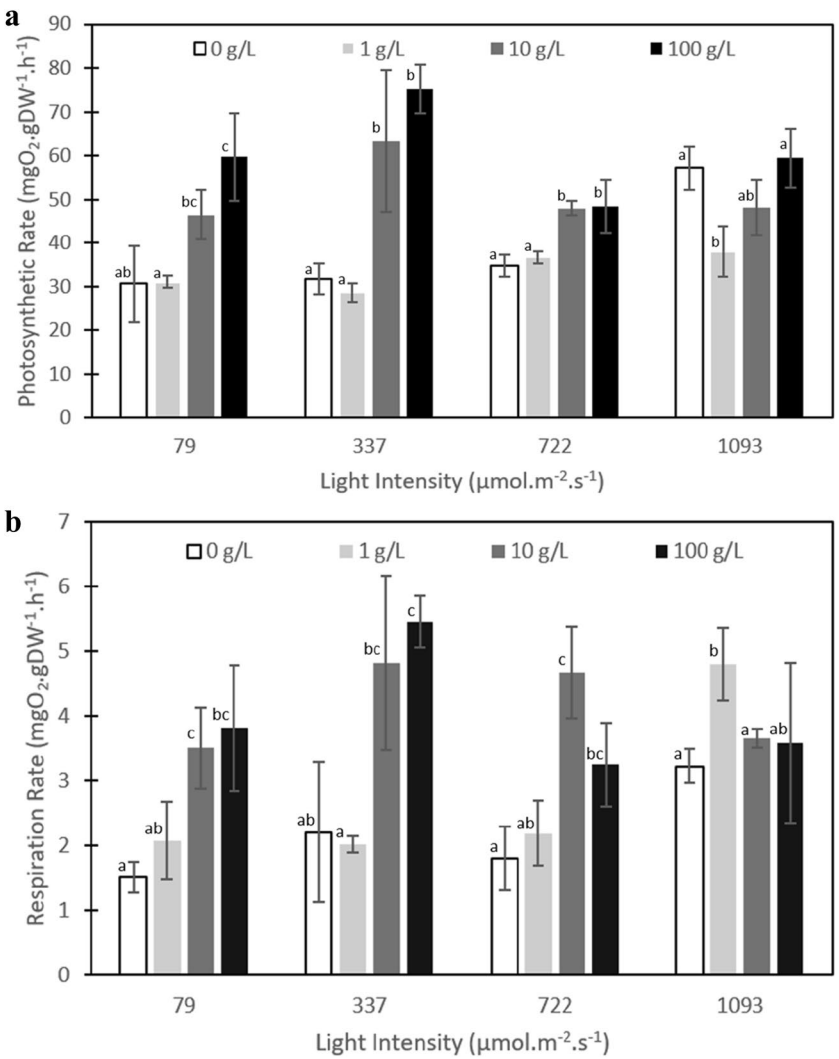

Fig. 3 Photosynthetic rate (a) and respiration rate (b) of *Tribonema* as a function of light intensity at different glucose concentrations. Data presented are the average and standard deviation of three experimental replicates (n = 3). Data within the same group that are not labelled with the same letter are statistically different (p <0.05)

mixotrophic samples. For the heterotrophic samples, the algae were quickly outcompeted by the actively metabolising bacteria and partially decomposed, making it non-harvestable at the end of the experimental period.

The inability to utilise acetate by *Tribonema* was an unusual finding, as many studies have documented that mixotrophic and heterotrophic algae that can use glucose can also utilise acetate as a carbon source (Bhatnagar et al. 2011; Wang et al. 2017; Patel et al. 2019). One possible reason for the different behaviour of *Tribonema* could be that the acetate molecule did not pass through the cell membrane. The utilisation of organic carbon by algae is usually via active transport into the cells, with various proteins and enzymes involved in the transport and metabolism of different organic molecules (Perez-Garcia et al. 2011). Importantly, the range and rate of organic carbon transport are usually species-specific depending on the presence of certain permeases (Bhatnagar et al. 2011). In the current experiment, the acetate did not appear to be transported into the cells, according to acetate concentration measurements performed before and after the experimental period that showed no decrease in the acetate level for the axenic samples. It is unclear whether the permeases for acetate are lacking in *Tribonema*, or if they were inhibited during the experimental period due to unknown reasons. Nevertheless, *Tribonema* may still be able to utilise acetate if it were transported across the cell membrane. It is not uncommon for obligate autotrophic algae to transition into heterotrophic mode when transporters for organic carbon molecules are introduced (Morales-Sánchez et al. 2015). The commonly reported metabolic pathways for acetate utilisation in algae involves conversion of acetate into acetyl-CoA as the first step, followed by further energy production or fatty acid synthesis (Perez-Garcia et al. 2011; Smith and Gilmour 2018). This is the same pathway as the utilisation of glucose following glycolysis. Since active glycolysis was evident in the experiments on glucose, it is likely that acetate can also be used by *Tribonema* metabolically. Therefore, the utilisation of acetate by *Tribonema* still

remains possible if the conditions for triggering production or removing inhibition of the corresponding permeases can be understood.

Ethanol

The results in Figures 2 and 3 show that ethanol provided no benefit to the productivity of *Tribonema* under any of the tested cultivation conditions. Interestingly though, a carbon mass balanced reveals that about 60% of the ethanol in the growth medium was consumed in both axenic experiments, despite no increase in productivity. This is likely attributable to an opposite situation compared to acetate: the ethanol molecules were transported into the algal cells but could not be beneficially utilised metabolically. Comparing the amount of ethanol consumed (5.9 – 6.2 mg) to the very limited increase in biomass DW for the mixotrophic axenic samples (1.9 – 3.0 mg), and the decrease in biomass DW for the heterotrophic axenic samples, it was unlikely that the ethanol was simply stored within the cells. Therefore, the ethanol that entered the cells must have been metabolised without positively contributing to algal growth. Ethanol has only rarely been reported as a cheap organic carbon source that may enhance the growth of some algal species (Sforza et al. 2012). However, it is generally more difficult to utilise than glucose or acetate and can be inhibitory to algal growth at high concentrations (John et al. 2011; Andemichael and Lee 2016). For the non-axenic cultures, measurements of the ethanol concentration in the growth medium showed full consumption occurred, indicating bacterial utilisation. Although this should have resulted in an increased CO_2 supply for the algae, no increase in productivity was observed. This was possibly due to ethanol inhibition of the algae (although not statistically significant, the productivity in the axenic mixotrophy cultures was 16% lower than in the autotrophy control) counterbalancing the beneficial benefits of bacteria-derived CO_2. Overall, these results suggest that ethanol is not a readily utilisable molecule for mixotrophic cultivation of *Tribonema*.

Glucose utilisation by *Tribonema*

Bacteria can outcompete Tribonema for glucose

While the axenic experiments (Figure 1) clearly show that *Tribonema* can successfully utilise glucose, the non-axenic experiments (Figure 2) and glucose utilisation data (Table 1) show that bacteria can outcompete the algae for glucose. Other studies have also identified rapid glucose consumption by bacteria and its impact on the mixotrophic and heterotrophic growth of algae. For example, bacteria sampled from lakes were found to have 10-30 times higher glucose uptake rate compared to *Chlorella protothecoides* and *Chlamydomonas acidophila* when grown separately (Kamjunke et al. 2008). When grown together with the algae using a chemostat, the algae were only able to survive in the presence of light (Kamjunke et al. 2008), similar to the observations found in the current study, in which *Tribonema* filaments were found to be highly friable after heterotrophic growth. Interactions between algae and bacteria must be considered in wastewater treatment since bacteria are abundant in the outdoor environment.

Algae and bacteria are generally considered to have a synergistic relationship due to gas exchange. Algal photosynthesis generates oxygen to support aerobic bacterial growth using the available organic carbon, which in turn provides CO_2 for algal photosynthesis (Jiang et al. 2021; Saravanan et al. 2021). However, studies have also shown inhibition of algal growth by bacteria under certain circumstances. For example, the productivity of *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* was found to be reduced in the presence of wastewater-borne bacteria unless extra glucose and nitrate supplementation was provided (Conceição et al. 2019). Such inhibition can be species-specific. For instance, *Bacillus pumilus* isolated from an algal production system was found to inhibit the growth of *Nannochloropsis* sp. by releasing certain inhibitory molecules, yet this did not affect the growth of other algal species such as *C. vulgaris* and *Tetraselmis striata* (Fulbright et al. 2016). Of potential relevance to the observations in the current study, this inhibition was initiated by an imbalance in the bacteria:algae ratio that could be related to the differential organic carbon utilisation rates of algae and bacteria. For example, organic carbon uptake by algae in lakes was found to be an order of magnitude lower than bacteria when the biomass density was an order of magnitude higher, indicating a dramatic difference in the organic carbon utilisation rate (Wright and Hobbie 1966). In addition, rapid organic carbon uptake may also result in increased water turbidity and further reduce the productivity of algae due to shading (Conceição et al. 2019). Depending on the complex growth conditions, the activity and the metabolic end products of bacteria can be beneficial or inhibitory for algal cultures (Li et al. 2023). To maximise algal productivity and wastewater treatment efficiency, the growth conditions should be carefully designed to maintain a stable ratio between algae and bacteria. This remains a complex yet important topic for future studies.

It is interesting that for the mixotrophic condition glucose consumption was significantly increased when bacteria were present (Table 1), yet the enhancement in areal productivity was similar to the axenic cultures (Figure. 2, Figure. 3). One explanation is that the increase in productivity is related to the rapid bacterial conversion of the glucose into CO_2, some of which is degassed before being utilised by the algae. As discussed above, bacteria can quickly outcompete algae in terms of glucose utilisation. In this case, as the glucose

present in the medium was converted to CO_2 by the bacteria, the original mixotrophic growth conditions revert to autotrophy. Since cultivation of filamentous algae in small culture flasks can be considered carbon-limited (Liu et al. 2023), an increased CO_2 level in the medium would increase algal productivity. As such, the lack of productivity enhancement despite the more significant glucose consumption under mixotrophic non-axenic conditions could be due to CO_2 off-gassing preventing much of the CO_2 generated from bacterial respiration being utilised during the experimental period, resulting in low carbon conversion. A further contributing factor could be that mixotrophic growth by *Tribonema* using glucose may reduce its CO_2 uptake rate during the Calvin Cycle in photosynthesis, possibly by production or substitution of some of the intermediate metabolic products.

Further explanation of the efficiency of carbon utilisation under the different modes of growth can be gained by comparing the extent of glucose removal from the growth medium to the biological carbon accumulation based on the change in algal dry weight (Table 1). Based on these results, differences in the major carbon fluxes under autotrophic, mixotrophic-axenic and mixotrophic non-axenic conditions can be proposed (summarised in Figure 4).

Interestingly, for the mixotrophic axenic condition the amount of carbon incorporated into the biomass was approximately equal to the amount of glucose-C consumed (Table 1). Therefore, by performing an overall carbon mass balance around the algal biomass, it can be inferred that the amount of inorganic carbon utilised by the algae over the experiment was approximately equal to the amount of CO_2 released due to respiration (refer to Figure 4). Since the cultures were cultivated under a day/night cycle, it can also be inferred that the net uptake of CO_2 during the day was approximately equal to the net amount of CO_2 released at night. This will be further discussed below. It is also worth pointing out that the values presented in Figure 4 represent the mass flux across the whole system. This resulted in an apparently complete conversion of glucose into new biomass under the mixotrophic, axenic condition. It is worth noting that this represents the net conversion, whereas in reality the CO_2 released during glucose metabolism was reutilised by *Tribonema* via photosynthesis in the carbon-limited environment (indicated by the dashed arrow). The amount of CO_2 involved in this reutilisation process was not quantitatively analysed in this study, which should be further addressed in future research.

For the mixotrophic non-axenic growth, as discussed in previous paragraphs, bacteria rapidly converted the available glucose into CO_2 which resulted in the growth mode leaning more towards autotrophy. Although algal productivity could still be enhanced to a similar level compared to axenic cultures, the carbon conversion rate was reduced due to potential CO_2 off-gassing. Carbon flux for the mixotrophic non-axenic growth is also presented in Figure 4.

Under heterotrophic, axenic conditions, about 37% of the glucose consumed was incorporated into the final biomass, equivalent to a biomass yield of about 0.33 g biomass g^{-1} glucose. This is lower than the reported typical range of about 0.4 – 0.6 g g^{-1} for heterotrophic growth of various algae using glucose (Shi et al. 1999, 2000; Jiang and Chen 2000). However, biomass yield is influenced by multiple factors such as glucose concentration, nutrient type and biomass concentration, and the current experiment was only designed to test the capability of heterotrophic growth for

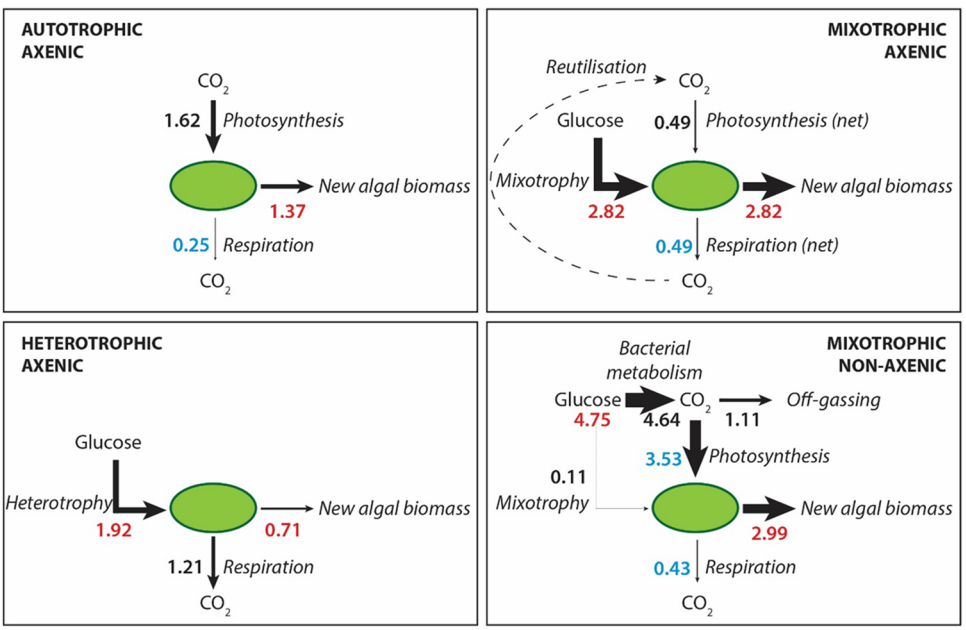

Fig. 4 Proposed summary of major net carbon fluxes in autotrophic (top-left), mixotrophic (top-right), heterotrophic (bottom left) & axenic, and mixotrophic & non-axenic (bottom-right) growth of *Tribonema* using glucose. The thickness of the arrows is scaled to indicate the relative amount of carbon in each pathway, based on the numbers obtained from Table 1 (red), calculated based on experimental respiration rates (Figure 3) and net biomass production (blue), and calculated using overall mass balance (black). The key assumptions and calculations are presented in Supplementary S3

Tribonema, therefore the growth conditions were not optimised. For example, it is possible that the oxygen present in the medium was insufficient to maintain strictly aerobic conditions for the duration of heterotrophic growth, leading to less efficient fermentation pathways under anaerobic conditions. Although the yield observed for *Tribomena* was somewhat lower than values reported for other algae, the results nonetheless confirm the ability to cultivate *Tribonema* heterotrophically.

Effect of glucose on algal photosynthesis and respiration

To provide more insight into the effect of glucose on *Tribonema* metabolism experiments involving direct measurement of dissolved oxygen were performed (Figure 3), which indicates the rate of light-dependent reactions during photosynthesis. This rate is usually determined by both the intensity of light (feedforward control) and the consumption of end products including ATP and NADPH within the Calvin cycle (feedback control) (Paul and Pellny 2003; Raines 2003). The effect of light intensity on photosynthesis can depend on various other factors such as species, temperature, nutrients, and the capability of the algae for adaptation by adjustment of the pigment level (Geider et al. 1998). However, in simple terms, low light intensity limits photosynthesis and high light intensity inhibits photosynthesis, with a plateau in photosynthetic efficiency at intermediate light levels (Béchet et al. 2013). In general, the optimal light intensity for most algal species falls within the range of 33 – 400 µmol photons m^{-2} s^{-1} for temperatures between 20 – 30 °C (Singh and Singh 2015). Few studies have specifically investigated the photosynthetic-irradiance response for *Tribonema*. Nevertheless, one previous study on a similar filamentous algae genus, *Oedogonium*, identified the optimal light intensity for photosynthesis to be about 75 – 500 µmol photons m^{-2} s^{-1} at 15 °C, with no photoinhibition observed for light intensity up to 1093 µmol photons m^{-2} s^{-1} (Pitawala et al. 2023). This agrees with another study that showed marginally increasing productivity of *Tribonema* with a light intensity increase from 60 to 480 µmol photons m^{-2} s^{-1} at 25 °C (Wang et al. 2016). It was also found that photoinhibition was less likely to occur at temperatures below 30 °C (Pitawala et al. 2023). Based on these findings from previous studies, it can be inferred that the experimental conditions in the current experiment (15 °C, 79 – 1093 µmol photons m^{-2} s^{-1}) should be appropriate for *Tribonema.* Nevertheless, the optimal temperature and light intensity ranges for *Tribonema* could differ from the ones applied in the current experiment. For example, as shown in Figure 3a, a minimal increase or even decrease in photosynthetic rate was observed at the higher light intensity ranges, which may indicate photo-inhibition. The optimal temperature and light conditions should be determined in future studies for further experimental optimisation.

In contrast to the effect of light intensity, which relates to feedforward control, the glucose concentration should affect the photosynthetic rate via a feedback control mechanism, with glucose metabolism providing a 'pull' to increase photosynthetic activity. To increase the photosynthetic rate, the glucose molecules first needed to be transported into the cells. The experimental results showed no apparent effect at 1 g L^{-1} glucose, but a strong dependence on the external glucose concentration at the higher range (10 and 100 g L^{-1}). This suggests that passive diffusion of glucose driven by extreme concentration gradients had a major influence on the results beyond that of active glucose transport. Glucose transport into algal cells usually follows active transport as the major pathway at lower concentrations, however passive and facilitated diffusion is gradient-dependent and can become more dominant at higher substrate concentrations (Wright and Hobbie 1966; Morales-Sánchez et al. 2017). In addition to the increased glucose transport rate, the glucose utilisation rate and flux through the Calvin cycle must also be increased in order to provide feedback control for the light-dependent reaction.

The general metabolic pathways and the means of organic carbon utilisation (glucose and acetate in particular) of algae have been reported in various literature and appear to be similar, regardless of species (Perez-Garcia et al. 2011; Morales-Sánchez et al. 2015; Smith and Gilmour 2018). A major pathway for glucose metabolism in algae is glycolysis, which converts glucose into acetyl-CoA that can be used for energy production or fatty acid synthesis. During glycolysis, glyceraldehyde-3-phosphate (GA3P) and 3-phosphoglycerate (3-PGA) are produced as key intermediates, both of which are also the key metabolic products of the Calvin cycle that are normally synthesised using CO_2 in autotrophy. When external glucose is provided for the algal growth, the level of GA3P and 3-PGA are expected to be high due to an increased level of glycolysis. This could in turn inhibit the upstream CO_2 uptake into the Calvin cycle, which helps to explain why the CO_2 uptake rate was reduced during the mixotrophy experiment. In comparison, other studies have shown the inhibition of organic carbon utilisation when excess CO_2 was provided to enhance photosynthesis for *C. protothecoides* and *Nannochloropsis salina* (Sforza et al. 2012). This further confirms the competitive relationship between the utilisation of glycolysis intermediates and CO_2 in the Calvin cycle.

The results presented here indicate that glucose taken up by *Tribonema* was used in glycolysis, generating GA3P and 3-PGA which inhibit the upstream CO_2 fixation in the Calvin cycle. However, it remains unclear whether and how the Calvin cycle functions with excess GA3P/3-PGA and reduced CO_2 intake, solely based on these productivity results. The

results from the photosynthetic activity experiments (Figure 3a) reveal an increasing photosynthetic rate as a function of glucose concentration, which implies an increase in ATP and NADPH consumption as a feedback control. Therefore, it can be inferred that the Calvin cycle, or at least the steps that involve consumption of ATP and NADPH, was still actively functioning, with the rate increasing as a result of the increasing glucose utilisation. One hypothesis based on the relevant metabolic pathways is referred to in the publication by Smith & Gilmour (2018). Briefly, they proposed that there exists a carbon loop between glycolysis and the Calvin cycle. During glycolysis, the metabolism of glucose follows the order of glucose – fructose-6-phosphate (F6P) – GA3P – 3-PGA. In reverse, the carbon flow in the Calvin cycle follows the order of 3-PGA – GA3P – ribulose-5-phosphate (Ru5P), which can then be converted back to F6P via the pentose phosphate pathway. This bypasses the intake of CO_2 during the Calvin cycle, while allowing ATP and NADPH consumption to provide feedback for the light-dependent reaction. However, there is no net gain of carbon or energy during this loop. The occurrence of this process may be solely due to the residual enzymes that remain during the relatively short experimental period, and the algal cells may actively adapt to more energy-efficient metabolic pathways if continuously exposed to glucose in the long term. Overall, an excessively high glucose content seemed to boost the photosynthetic rate of *Tribonema*, however more detailed analysis such as tracked carbon-14 experiments would be required to understand the mechanism and how this trend can be optimised for long-term cultivation.

The trends for the respiration rate (Figure 3b) are very similar to the observations found for the photosynthetic rate. The respiration rate measured in this experiment consists of both the dark/basal respiration and the light-enhanced respiration (Pitawala et al. 2023). The change in light intensity mostly affected the light-enhanced respiration rate. As previously discussed, the experimental conditions provided for this experiment represent a light intensity range close to optimum with no photoinhibition. The similar trend observed for the respiration rate further confirms this finding as the light-enhanced respiration rate is highly related to the light intensity. On the other hand, the glucose present may have enhanced both the dark respiration and the light-enhanced respiration. Organic carbon provides both energy and important carbon substrates for mixotrophic algal growth (Bouarab et al. 2004). Energy is transmitted in form of the acetyl-CoA produced from glycolysis that can be utilised in the TCA cycle for energy production. With the level of glucose increased, the oxygen required for energy production would also increase, leading to an enhanced level of dark respiration. In addition, the presence of glucose would also result in key metabolic intermediates and trigger higher levels of photosynthesis, as previously discussed. This may also boost the level of light-enhanced respiration as the respiration rate was measured shortly after the illuminated period in the current experiment.

Conclusion

Tribonema was found to directly utilise glucose for both mixotrophic and heterotrophic growth, representing a reduction in the CO_2 requirements. In contrast, acetate could only be indirectly utilised mixotrophically with the presence of bacteria, whereas ethanol could not be utilised. Under non-axenic conditions, the organic carbon consumption rate of bacteria seemed to be dominative over algae. Further experiments confirmed the enhancements to the metabolic pathways by glucose utilisation, however the detailed mechanism remains unconfirmed experimentally. Although *Tribonema* showed some positive results in its ability to utilise glucose, direct utilisation of organic carbon in wastewater is unlikely to play a significant role as the bacteria can outcompete the algae for glucose. As such, bacterial utilisation of organic carbon to provide CO_2 will be more important to supplementing algal growth. Nonetheless, the ability to utilise glucose could be applicable to axenic mixotrophic cultivation of filamentous algae such as *Tribonema* for higher-value applications.

The study indicates the involvement of bacteria is important and that optimisation of algal-bacteria interactions is still required, especially for large-scale outdoor cultivation. The bacterial and organic composition of wastewater is usually very complex and hard to control, especially for high-strength upstream wastewater. Given that *Tribonema* was easily dominated by bacteria in the current experiments, especially during dark periods, the use *Tribonema* appears better suited for treatment of partially treated wastewater with low concentrations of organic carbon.

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Authors' contributions J. Liu developed and conducted the experimental program, interpreted the results, drafted, revised and approved the final manuscript. N. Crosbie and P. Scales contributed to the conceptual development and interpretation of the research, and revised and approved the final manuscript. G. Martin assisted in the design of the experimental program, conceptual development, data interpretation,

and preparation of the manuscript. G. Martin takes responsibility for the integrity of the entire work and can be contacted as gjmartin@unimelb.edu.au.

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Data availability The data supporting the findings of this work are available within the paper and its Supplementary Information file. The algae strain is maintained at The University of Melbourne. Should the algae strain or any raw data be needed in another format they are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors have no competing interests to declare.

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