Determination of Reliable Biomass Indicators in the Cyanobacterium Gloeothece

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ABSTRACT: *Gloeothece* is a nitrogen-fixing cyanobacteria surrounded by a polysaccharide sheath. The sheath has provided problems with determining the biomass for this species and so a number of techniques were tested to find the most reliable method. Optical density, chlorophyll a, protein and phycobiliprotein concentration were all measured every 2 weeks for a period of 6 weeks. These were compared with biovolume, which was calculated by determining cell abundance and mean cell volume from images taken using Laser Scanning Confocal Microscopy and imaging software. Two strains of *Gloeothece* were studied (the wild type strain with a sheath, and mutant strain without a sheath) to provide comparisons between sheath bound and sheathless organisms. The results showed that optical density correlated well with biovolume for both strains of *Gloeothece* (wild type and mutant $p = <0.001$). Both protein and chlorophyll a concentrations showed strong correlations with biovolume in the wild type strain (protein $p = 0.001$; chlorophyll a $p = 0.03$) but not the mutant. Phycobiliprotein concentration showed highly variable results, the comparisons of 3 pigments were used (PC/AP and AP/PE ratios) showing no significant correlations for PC/AP ratios, but correlations with biovolume for AP/PE ratios for both strains (wild type $p = 0.008$; mutant $p = 0.002$). From this study it can be concluded that measurement of optical density is the most reliable indicator of biomass for *Gloeothece*. This method could potentially be applied to other sheath bound organisms.

Keywords: Biomass, Biovolume, Cyanobacteria, Laser Scanning, Confocal Microscopy, Sheath.

1. INTRODUCTION

Cyanobacteria are important organisms found in a range of freshwater and marine environments and certain extreme environments such as hot springs and Antarctic lakes [1, 2, 3]. *Gloeothece* is a unicellular cyanobacterium commonly found attached to calcareous rocks, stones in freshwater and are surrounded by a relatively inner transparent sheath enclosing 1-2 cells and an outer opaque sheath enclosing groups of cells [4]. The presence of a sheath makes determining the biomass for these cells problematic. In previous studies a number of methods have been used for measuring biomass in cyanobacteria which include Utermöhl sedimentation technique [5, 6], chlorophyll a concentration [7], turbidity [8], dry mass [9,10] and calculating biovolume [11], phycocyanin fluorescence [12]. All of these techniques have provided reliable methods for these organisms. In certain cases, it may be necessary to determine the suitability of the methods used to a particular type of microorganism under study [13].

The classical methods for quantifying cells such as flow cytometry (FCM), used commonly for counting unicellular organisms including many species of phytoplankton can provide inaccurate calculations of biomass since FCM deals with particles of varying size between 0.5 and 30 mm and particle numbers ranging from 102 to 106 per ml [14]. For species that produce colonies, the increase in the size range 10 to 100 fold may affect the accuracy and precision of the result and may require the determination of relation between colony size and cell numbers [15, 14]. FCM would also provide inaccurate calculations of biomass of *Gloeothece* because a group of cells within a

sheath would be counted as one and another reason is that the number of cells colonised within the sheath does not stay constant, therefore the estimation of number of cells would be highly inaccurate. Methods that are based on optical and electron microscopy require manipulation of the sample. This manipulation limits the accuracy mainly because the reduction in cellular biovolume and the sample dilution [16]. Fluorescence microscopy may also be used; however the images obtained show high 'out-of-focus' fluorescence as documented by Sole et al. (2001) [17]. The concentration of chlorophyll a within the cells is a common technique used for estimating phytoplankton abundance. The technique is often criticised, however, because of inefficient extraction methodology and variability in the chlorophyll a content of phytoplankton cells [18]. It has been noted that chlorophyll fluorescence data usually correlates linearly with cell numbers for a given set of environmental conditions [19]. Protein concentration has not been widely used as an indicator of biomass in phytoplankton. The protein concentration measured using the Lowry method is constant in its sensitivity to different proteins providing absolute concentrations of proteins and therefore may provide as a useful technique in biomass determination. Turbidity has also been used as a successful indicator of biomass in previous studies [20, 21]. However the sheath of Gloeothece may alter the accuracy of this technique, as it may affect the absorbance of the sample. Different pigment ratios of three phycobiliprotein pigments: Phycoerythrin (PE), Allophycocyanin (AP) and Phycocyanin (PC) can an also be used to provide an estimate of biomass. This technique has not been so widely used but has provided successful results in quantification in the number of cyanobacteria in water samples [22].

This study focuses on several methods that have been previously used to quantify cell abundance and biomass in phytoplankton including calculating biovolume with the use of confocal microscopy; Chlorophyll a concentration; Protein concentration; Turbidity and Phycobiliprotein concentrations to determine reliable indicators of biomass for *Gloeothece*. The investigation will be relevant for the determination of biomass for other sheath bound and mucus embedded phytoplankton such as Phaeocystis and Anabaena.

2. MATERIALS AND METHOD

Two strains of *Gloeothece* were used for comparisons, the wild type (PCC 6909) that has a sheath, and the mutant (PCC 6909-1) which does not have a sheath. The study was carried out over a period of 6 weeks with measurements for chlorophyll a, protein, phycobiliprotein and turbidity taken every 2 weeks. For each replicate (3 replicates) 10 images were taken every 2 weeks using laser scanning confocal microscope and imaging software. Measurements were taken every 2 weeks due to the slow growing nature of *Gloeothece*. Both the wild type and mutant strains of *Gloeothece* were cultured in 30 ml BG-110 (nitrate omitted) medium and left for 2 weeks before the first extraction was taken.

2.1. Chlorophyll a Determination:

Samples from wild type (3 ml) and mutant (10 ml) were centrifuged at 10,000 g for 10 min. The supernatant was removed and the cells were resuspended in 1 ml of 90 % methanol. Samples were left at 4 °C in dim light for 24 h after which samples were re-centrifuged at 10,000 g for 10 min. Absorbance of the supernatant was measured at OD665. The chlorophyll concentration was measured using the following equation:

Chl a (µg ml-1) = OD665nm * 13.9 (Chl a coefficient)

Which gave the concentration of chlorophyll a in the volume of sample originally extracted from after which the concentration per millilitre was calculated.

2.2. Phycobiliprotein Determination:

The cells were broken in order to extract the phycobiliproteins. Samples were taken for extraction from the wild type (4.5 ml) and mutant (10 ml) which was centrifuged at 10,000 g for 10 min. The supernatant was

removed and the cells were resuspended in 1 ml 20 mM sodium acetate buffers at pH 5.5. The cells were then broken in a French Cell Press at 1000 psi. 200 µl of 2 % streptomycin sulphate was added to extract the phycobiliproteins. The samples were left for at least 24 h at 4 °C and then re-centrifuged at 10,000 g for 10 min and the absorbance of the supernatant was measured at OD565, OD620 and OD650. The concentrations of Phycocyanin (PC), Allophycocyanin (AP) and Phycoerythrin (PE) were determined according to technique described by Tandeau de Marsac and Houmard (1988) [23].

PC (mg ml-1) = OD620 – 0.7 * OD650 / 7.38

AP (mg ml-1) = OD650 – 0.19 * OD620 / 5.65

PE (mg ml-1) = OD565 – 2.8[PC] – 1.34[AP] / 12.7

This gave the concentration of PC, AP and PE in the volume of sample originally extracted from after which then concentration per millilitre was calculated for final results.

2.3. Optical density:

For determination of optical density 2 ml of the sample of both the wild type and mutant was taken. The samples were shaken and then the absorbance was measured at OD_{436}.

2.4. Protein Determination:

Samples were centrifuged at 10,000 g for 10 minutes at 4°C. The pellet was resuspended in 100 µl 1N NaOH and hydrolysed at 100 °C for at least 10 minutes. Protein concentration was estimated according to Lowry method [24].

2.5. Cell Biovolume:

Biovolume was calculated by taking 10 images per replicate using Zeiss LSM Image Examiner and Laser Scanning Confocal Microscope. The objective used is Plan-Neofluar 40x/1.3 Oil DIC and a 488 nm laser was used to excite the chlorophyll a. Samples were diluted and placed in a 2 ml settling chamber, the images were taken in random areas around the settling chamber. The cells that were completely within the image are counted, along with the cells that were partly off the image on the top and right side. Cells partly off the left and bottom sides of the image were not counted. The abundance for each sample was calculated by multiplying the number of cells counted with the fraction of the settling chamber examined, the dilution factor was then accounted for and the sample volume and the number of cells per ml was calculated. For the wild type strain z-stacks were taken. Z-stacks provide a 3 dimensional view of the sample as a number of images were taken at 25 µm – 40 µm (top-bottom distance) slices through the sample, depending on the requirements for the sample i.e. the number of cells. This was to ensure that all cells were counted as with the wild type strain the cells may orientate themselves on top of each other within the sheath.

The cell volume was also calculated using the images taken. The length and diameter were measured ensuring the cells are orientated the correct way and that the cells are an oval shape. Fifty cells per replicate were measured, which is approximately 5 cells per image. The cell volume was then calculated according to Sun and Liu (2003) by the following equation [25]:

Cell Volume (µm3 ml-1) = (π * length * diameter2) / 6

Biovolume was calculated by multiplying cell volume with cell abundance.

2.6. Statistical Analysis:

The results were statistically analysed, the parameters were correlated with biovolume and the significance of the relationship was tested with Pearson's Correlation Coefficient, with a significant correlation having a critical p-value <0.05. The change of cell abundance, and biovolume was tested with a one-way ANOVA, with a significant p-value <0.05.

3. RESULTS

Cell abundance and cell volume were calculated from images collected from laser scanning confocal microscope and image software. The abundance of both strains was studied over the 6 weeks period (Fig. 1a). A significantly higher number of cells was found in the wild type strain compared to the mutant strain (ANOVA p = <0.001). Also an increase in the number of

cells was observed throughout the study period (mutant ANOVA p = 0.008; wild type ANOVA p = <0.001).

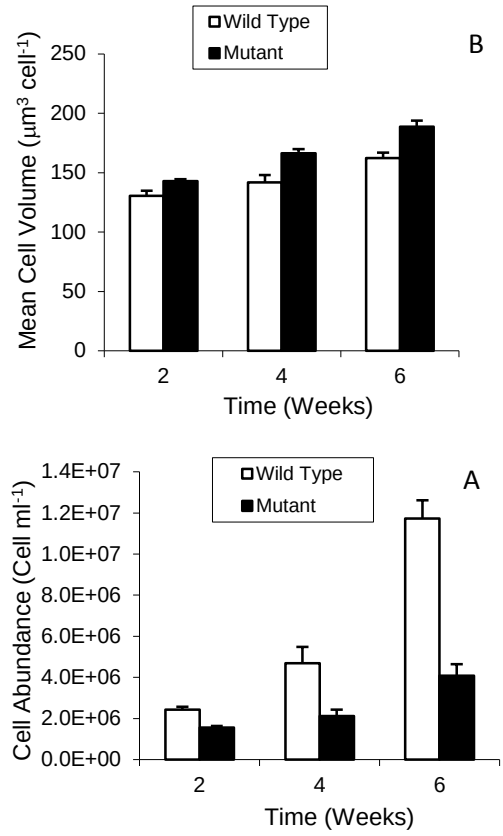


Figure 1: a) Change in cell abundance for wild type and mutant species of *Gloeothece* over a 6-week study period (± SE). b) Change in mean cell volume (MCV) for 2 species of *Gloeothece* over a 6-week study period (± SE).

From Fig. 1b it is clear that the mean cell volume of the mutant cells is larger than the wild type cells (ANOVA p¬ = <0.001). There is a slight increase of the mean cell volume over the 6-week period. The one-way ANOVA gave significant values of <0.001 for mutant and 0.014 for wild type; therefore it is fair to say that this increase is not an anomaly and that over time the cells increase in volume.

Change in biovolume (Fig. 2) over the 6-week study period shows the wild type strain has a significantly higher biovolume compared to the mutant strain (ANOVA p = <0.001) due to the higher cell abundance. The biovolume increases over time for both species (mutant: p-value = 0.02; wild type: p-value = <0.001) due to the increase in cell abundance and mean cell volume.

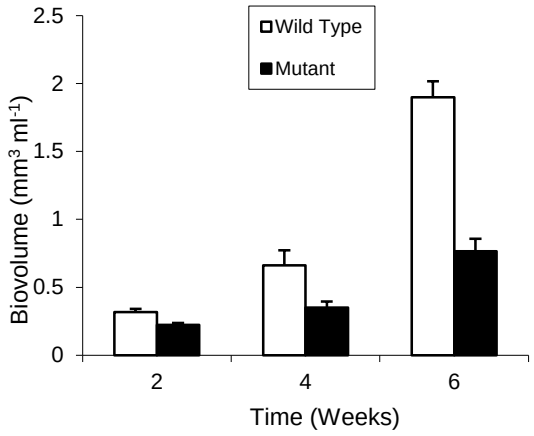
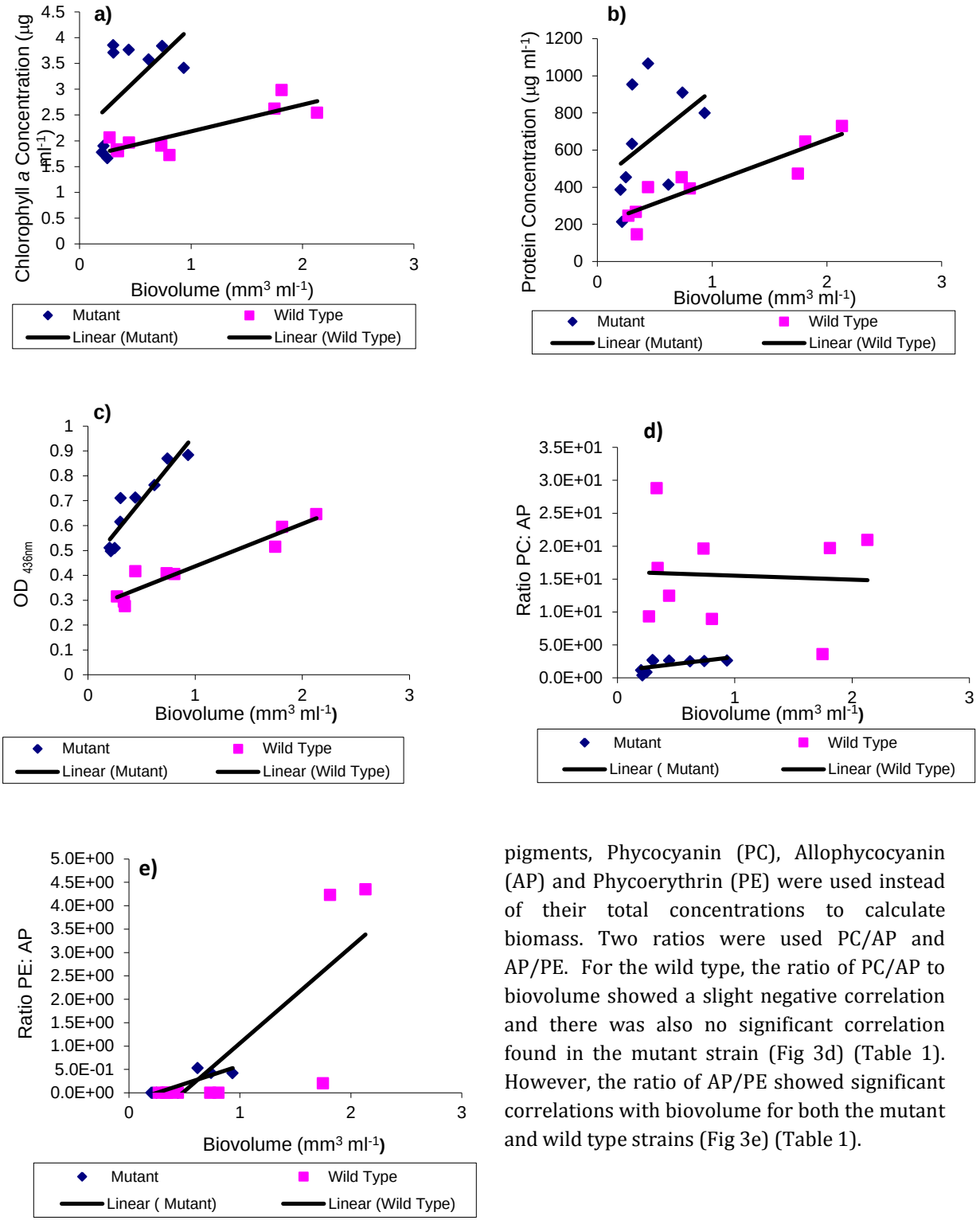


Figure 2: Change in biovolume in both wild type and mutant strains of *Gloeothece* over a 6-week study period (± SE).

The biovolume was used to correlate with the other parameters to test the reliability of them as indicators of biomass. Fig. 3a-e shows the correlation between optical density, chlorophyll a, protein, and phycobiliprotein concentration and the biovolume of the cells. Fig. 3a shows that for the mutant strain chlorophyll a content correlates poorly with biovolume, whereas the wild type strain show a significant correlation (Table 1). The correlation between protein concentration and biovolume is shown in Fig. 3b. As with chlorophyll a the wild type strain shows a good correlation. The mutant strain however, has a highly variable correlation between protein concentration and biovolume (Table 1). The optical density measurements show strong correlations for both the mutant and wild type strains (Fig. 3c; Table 1) and therefore could be used as reliable indicators of biomass for both strains of *Gloeothece*. Comparisons of three phycobiliprotein



pigments, Phycocyanin (PC), Allophycocyanin (AP) and Phycoerythrin (PE) were used instead of their total concentrations to calculate biomass. Two ratios were used PC/AP and AP/PE. For the wild type, the ratio of PC/AP to biovolume showed a slight negative correlation and there was also no significant correlation found in the mutant strain (Fig 3d) (Table 1). However, the ratio of AP/PE showed significant correlations with biovolume for both the mutant and wild type strains (Fig 3e) (Table 1).

Figure 3: Correlation of 2 strains of *Gloeothece* between the biovolume and a) Chlorophyll a; b) Protein; c) Optical density (OD); d) Ratio of phycobiliproteins PC:AP e) Ratio of phycobiliproteins AP:PE

Table 1: Statistical analysis testing the significance of correlations between the parameters chlorophyll a, protein, optical density and phycobiliproteins with biovolume. *p-value <0.05 = correlation is significant.

Parameter	Strain	Equation of the Line	Pearson's Correlation Co-efficient (r)	Significance (p)	Number of Variables (n)
Chlorophyll a	Wild Type	y = 0.5178x + 1.6642	0.855	0.03*	9
	Mutant	y = 2.0727x + 2.132	0.561	0.116	9
Protein	Wild Type	y = 229.51x + 197.31	0.899	0.001*	9
	Mutant	y = 493.8x + 427.56	0.434	0.243	9
Optical density	Wild Type	y = 0.1706x + 0.2661	0.954	<0.001*	9
	Mutant	y = 0.5318x + 0.437	0.925	<0.001*	9
Phycobiliprotein ratio PC:AP	Wild Type	y = -0.5951x + 16.129	-0.065	0.868	9
	Mutant	y = 2.1285x + 1.0544	0.608	0.083	9
Phycobiliprotein ratio AP:PE	Wild Type	y = 2.0573x - 0.9984	0.810	0.008*	9
	Mutant	y = 0.7682x - 0.1904	0.873	0.002*	9

4. DISCUSSION

This study focused on *Gloeothece*, nitrogen fixing freshwater cyanobacterium which has been studied in several areas, including nitrogen fixation, quorum sensing, bioremediation etc. [26, 27, 28, 29]. *Gloeothece* have a polysaccharide sheath surrounding individual and groups of cells [4]. The sheath resulted in the determination of biomass problematic, with some methods providing inaccurate results. This study looked at a number of ways to measure the biomass in order to determine the most reliable indicator and used two strains of *Gloeothece* to provide comparisons.

Although the method to calculate biovolume through Confocal laser scanning microscopy was found to be time consuming it has produced useful results to compare with the other parameters. The technique is useful as it allows a large number of samples to be analysed quickly and easily [16]. For the wild type strain the identification of stacked cells may prove to be problematic. Carbon conversion factors that are applied to biovolume in order to calculate biomass [6, 5] may not become useful for *Gloeothece* as the sheath contains unknown amounts carbon and further investigations would be needed to determining the carbon conversion factors.

The relationship between chlorophyll a and biovolume for the wild type strain was found to be significant and correlated well. For the mutant strain, no significant correlation was found with the biovolume. This could be related to physiological differences between the mutant and wild type strain which could depend on factors such as light and nutrients which are known to affect chlorophyll a concentration [14]. The technique of extracting the chlorophyll a has also been criticised in

previous studies [17]. Therefore, the high variability between the stains may not only be with the method but also with the external factors effecting the concentration and of chlorophyll a.

As with the chlorophyll a, wild type protein concentration showed a significant correlation with biovolume, whereas the mutant did not. It has been found that many substances used in culture media may interfere with the Lowry method and can cause changes in the value of the absorbance of the sample [30] and could be a factor responsible for the poor correlation between protein concentration and biovolume in the mutant strain.

Optical density has been used as an indicator of biomass in numerous studies, mainly for quantifying bacteria [31]. It was assumed that the sheath would have a variable effect on the absorbance; however this has not found to be the case, possibly due to the transparency of the sheath. The results also showed mutant strain cultures to be more turbid than the wild type cultures, which was not expected especially as there were fewer cells in the mutant samples and could be due to the higher mean cell volume seen in the mutant strain or the lack of sheath. This method was found to be simple and a reliable method for calculating biomass in Gloeothece.

Rapala et al., (2002) used phycobiliprotein pigment ratios as a reliable method of determining biomass, correlating with heterotrophic plate counts. A similar method was used in this study and used in comparisons of pigments (PC:AP and AP:PE ratios) rather than total concentrations to determine biomass. It has been found that the content of PE pigment is higher as a response to low light [32]. Therefore, changes in light availability and intensity can affect the concentration of phycobiliprotein pigments, which may occur as cell density increases. The comparison of pigments PC: AP showed no correlation with biovolume in either of the strains of Gloeothece. The comparison of pigments AP:PE however, showed significant correlations with biovolume for both the wild type and the mutant strains. The extraction of phycobiliproteins, like the chlorophyll a method can prove inaccurate, especially as a loss of cells may occur when they are broken. A sufficient number of cells and volume of sample was required to produce a blue colour to the sample in which the absorbance was measured. Although comparisons of AP:PE showed a significant correlation with biovolume, inaccuracies within the method may lead to inaccuracies in the results and using this method for determining the biomass of both strains of *Gloeothece* or other sheath bound cells may need further investigation.

5. CONCLUSION

Overall the comparison between the two strains of *Gloeothece* showed similarities and differences when determining the biomass. Three of the four parameters for the wild type strain correlated well with biovolume. Although chlorophyll a showed a strong correlation, the gradient of the line was shallow and gave a small variability in concentration compared to the relatively large change in biovolume over the study; this would therefore make it less reliable as an indicator of biomass. Protein and optical density measurements showed the most significant correlations and therefore these methods would be a reliable way of determining biomass for the wild type strain of *Gloeothece*. For the mutant strain, only optical density correlated well with biovolume, therefore, would prove to be a reliable indicator of biomass for this strain. Although the other parameters did not correlate well, due to the absence of the sheath, methods such as flow cytometry could be used to quantify biomass in this strain.

There are a number of issues linked to this study that can be further investigated. Carbon conversion factors for *Gloeothece* may provide useful in determining the biomass and could be applied to biovolume data. Phycobiliprotein pigments also need further investigation as an indicator of biomass, as this study showed highly variable results, looking at whether the ratio AP:PE pigments could be used. Computer-assisted programs for quantifying cell abundance using confocal microscopy could also be investigated, testing whether stacked cells within the sheath would prove problematic.

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