

# Enhanced Production of Green Tide Algal Biomass through Additional Carbon Supply

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## Abstract

Intensive algal cultivation usually requires a high flux of dissolved inorganic carbon (Ci) to support productivity, particularly for high density algal cultures. Carbon dioxide (CO<sub>2</sub>) enrichment can be used to overcome Ci limitation and enhance productivity of algae in intensive culture, however, it is unclear whether algal species with the ability to utilise bicarbonate (HCO<sub>3</sub><sup>-</sup>) as a carbon source for photosynthesis will benefit from CO<sub>2</sub> enrichment. This study quantified the HCO<sub>3</sub><sup>-</sup> affinity of three green tide algal species, *Cladophora coelothrix*, *Cladophora patentiramea* and *Chaetomorpha linum*, targeted for biomass and bioenergy production. Subsequently, we quantified productivity and carbon, nitrogen and ash content in response to CO<sub>2</sub> enrichment. All three species had similar high pH compensation points (9.7–9.9), and grew at similar rates up to pH 9, demonstrating HCO<sub>3</sub><sup>-</sup> utilization. Algal cultures enriched with CO<sub>2</sub> as a carbon source had 30% more total Ci available, supplying twenty five times more CO<sub>2</sub> than the control. This higher Ci significantly enhanced the productivity of *Cladophora coelothrix* (26%), *Chaetomorpha linum* (24%) and to a lesser extent for *Cladophora patentiramea* (11%), compared to controls. We demonstrated that supplying carbon as CO<sub>2</sub> can enhance the productivity of targeted green tide algal species under intensive culture, despite their clear ability to utilise HCO<sub>3</sub><sup>-</sup>.

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## Introduction

Macroalgal biomass is an emerging resource for sustainable bioenergy [1] and advanced biofuels [2,3]. Bioenergy applications rely on the production of a high volume/low value biomass opening opportunities to develop the culture of new commercial species. Green tide algae have the potential to meet these criteria as they are fast growing species [4] with a tolerance to a broad range of environmental conditions [5]. Furthermore, they are highly suitable as a bioenergy feedstock for ethanol [6], biogas [7,8] and thermo-chemical conversion to biocrude [3,9]. Green tide algae can be cultured extensively in open water culture [10] or harvested from natural blooms [11]. Alternatively, they can be cultured intensively in land-based ponds and tanks integrated into nutrient-rich aquaculture [12–14] and municipal [15] waste streams for bioremediation.

Dissolved inorganic carbon (Ci) is usually the limiting factor for growth in intensive cultivation with nutrient-rich systems, as the rate of Ci assimilation by the algae is greater than the rate of CO<sub>2</sub> diffusion from the air into the water, even when vigorous aeration is used [16]. Total Ci in the water is composed of an equilibrium between carbon dioxide (CO<sub>2</sub>), bicarbonate (HCO<sub>3</sub><sup>-</sup>) and carbonate (CO<sub>3</sub><sup>2-</sup>), which are part of a buffered system. The relative amount of each fraction is dependent on pH, and to a lesser extent on salinity and temperature [17]. At pH 6, the molar fraction of the total Ci is divided equally between CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>, the only usable forms of carbon for most of algae. The

concentration of CO<sub>2</sub> at pH 8.5 is negligible, as HCO<sub>3</sub><sup>-</sup> is in equilibrium with CO<sub>3</sub><sup>2-</sup>, which is not a direct source of inorganic carbon for algal photosynthesis [18]. Above pH 9, the relative fraction of HCO<sub>3</sub><sup>-</sup> continues to decrease relative to CO<sub>3</sub><sup>2-</sup> leading to Ci limitation. Ironically, the daily pH fluctuations in a carbon limited system of intensive algal cultivation can cross at least 2 pH units [19], which provide a unique setting to evaluate the benefits of dosing Ci at commercial scales.

Increasing total dissolved CO<sub>2</sub> concentrations in land-based intensive seaweed cultivation can therefore significantly enhance biomass productivity [20–23]. However, CO<sub>2</sub> enrichment can also have no effect or may even be detrimental for some species [24–26]. The lack of widespread positive responses to CO<sub>2</sub> enrichment in algae has been attributed to the presence of carbon concentration mechanisms (CCMs). These mechanisms allow algae to utilize the HCO<sub>3</sub><sup>-</sup> pool in seawater, which is the most common form of carbon [27]. However, the efficiency of HCO<sub>3</sub><sup>-</sup> use is species specific, with some species relying on HCO<sub>3</sub><sup>-</sup> to complement CO<sub>2</sub> as a carbon source, while others can efficiently saturate carbon requirements using HCO<sub>3</sub><sup>-</sup> alone [28]. Therefore, enhanced productivity through CO<sub>2</sub> enrichment is affected by the capability and efficiency with which species use HCO<sub>3</sub><sup>-</sup>. Quantifying and understanding the response of algae to CO<sub>2</sub> enrichment is a critical first step in optimisation of growth under intensive culture.

The major objective of this study was to quantify the ability of three green tide algal species, *Cladophora coelothrix* Kützting,

*Cladophora patentiramea* (Montagne) Kützing and *Chaetomorpha linum* (O. F. Müller) Kützing, to utilise alternative carbon sources under intensive culture, and subsequently quantify their growth response to removing carbon limitations. These three species were selected as they are clearly identified targets for biomass production for bioremediation and bioenergy applications in tropical Australia [14,29], and worldwide [8,10,30]. Specifically, we quantified the affinity for  $\text{HCO}_3^-$  as a carbon source using the pH drift technique to determine the compensation point. We subsequently quantified growth under laboratory conditions at different pH levels with defined carbon sources. Finally, the three species were cultured for four weeks in an outdoor experiment testing the effects of  $\text{CO}_2$  enrichment and  $\text{HCO}_3^-$  affinity on productivity and elemental composition (carbon, nitrogen and ash).

## Materials and Methods

### Algae collection and stock cultures

Three green tide algal species were collected from private aquaculture facilities in Queensland, Australia. *C. coelothrix* and *C. linum* were collected from the settlement pond and intake channel, respectively, of an intensive fish farm (Latitude: 20.02°S Longitude: 148.22°E, barramundi *Lates calcarifer*). *C. patentiramea* (Montagne) Kützing was collected from the intake dam of an intensive prawn farm (Latitude: 18.26°S Longitude 146.03°E, tiger prawns *Penaeus monodon*). Permission was obtained from owners to collect algae from these sites. Algal samples were hand collected and placed in aerated seawater for transportation to the James Cook University, Marine Aquaculture Research Facility Unit (MARFU). Stock cultures of each algal species were maintained in 70 L tanks within a recirculating system (~27°C, 36‰).

### Algal affinity for $\text{HCO}_3^-$

Two approaches were used to quantify the ability of the three algal species to utilize  $\text{HCO}_3^-$  as a source of Ci; pH drift technique (compensation point), and algal growth response to different pH levels.

### pH drift in closed vessel

The pH drift technique is a reliable method to determine  $\text{HCO}_3^-$  utilization [31]. As the photosynthetic uptake of  $\text{CO}_2$  and/or  $\text{HCO}_3^-$  results in a near stoichiometric production of hydroxyl ions, the pH of the culture media increases in response to photosynthesis. At pH 9, dissolved  $\text{CO}_2$  is virtually absent and species without mechanisms of  $\text{HCO}_3^-$  utilization reach their limit of Ci extraction. Consequently, pH will not increase beyond this level, enabling the ability to utilise  $\text{HCO}_3^-$  to be evaluated [32].

The pH drift assays were carried out in a culture chamber (Sanyo model MLR-351) with constant temperature (28°C) and irradiance (150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Basal culture media was prepared using filtered sterile seawater ( $\text{NO}_3\text{-N}$  0.06  $\text{mg l}^{-1}$ ,  $\text{PO}_4\text{-P}$  0.02  $\text{mg l}^{-1}$ , Ci 1.9 mM and 32 ‰) enriched with f/2 growth media [33]. Algal samples were collected from the stock cultures, washed clean and pre-incubated for five days in the conditions described above. Approximately 100 mg fresh weight of filaments were incubated in closed airtight 120 ml graduated culture vessels filled with 130 ml of freshly prepared growth media (pH 7.9), leaving a minute air space. Culture vessels were repositioned and stirred hourly during the experiment to minimise any artefacts relating to light source or the formation of a boundary layer.

Thirty-six culture vessels were prepared for each species and three random culture vessels for each species ( $n = 3$ ) were destructively sampled for pH measurements (YSI 63 pH meter).

The pH drift assays ran for twelve hours. The pH measurements were performed at one and two hours in culture and then repeated every two hours until the maximum pH reached a stable level for at least two consecutive measurements (pH compensation point). This compensation point represents the pH at which the Ci taken up by the algae equals the  $\text{CO}_2$  released by respiration and/or photorespiration into the medium.

### Effects of pH on algal growth

The  $\text{HCO}_3^-$  affinity of the algae can be inferred from their growth response at different pH levels because the relative amount of  $\text{CO}_2$  and  $\text{HCO}_3^-$  available for growth is pH dependent. Above pH 8.5, where  $\text{CO}_2$  is virtually absent, species with no or little ability to use  $\text{HCO}_3^-$  experience a steep decrease in growth. In contrast, species with the ability to efficiently use  $\text{HCO}_3^-$  respond more slowly to the increase in pH as they utilize  $\text{HCO}_3^-$  for growth.

To test  $\text{HCO}_3^-$  affinity, algal biomass was transferred from the outdoor stock cultures to the laboratory and pre-cultured in f/2 enriched growth media for five days (in conditions described in the previous section). The growth experiment was carried out in a culture chamber (Sanyo model MLR-351) with constant temperature (28°C) and irradiance (150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) with a 12 L:12 D photoperiod. Samples of each algal species (~100 mg fresh weight) were incubated in 100 mL of seawater enriched with f/2 growth media [33] within 120 mL plastic culture vessels with the lid loosely placed on top. The culture media in each treatment was buffered to maintain constant pH (+ 0.1 units), and correspondingly Ci ratios, using biological Tris (Sigma) at a final concentration of 25 mM. The water pH was adjusted to the desired pH levels (7, 7.5, 8, 8.5 and 9) using freshly prepared 1 M NaOH or HCl solutions. The culture media was prepared and replaced every day to renew Ci and to maintain the original  $\text{CO}_2\text{:HCO}_3^-$  ratios for each treatment. Algal filaments were filtered through a mesh screen and resuspended in the new growth media. Samples were again stirred and repositioned daily to a new position in the culture chamber. Treatments were weighted at the beginning and end of a ten day experimental period. Daily growth rates (DGR; %  $\text{day}^{-1}$ ) were then calculated using the following equation:

$$\text{DGR} = \left[ \left( \frac{\text{Wf}}{\text{Wi}} \right)^{(1/T)} - 1 \right] * 100 \quad (1)$$

where Wi is the initial fresh weight, Wf is the final fresh weight and T is the culture period in days.

### Algal productivity under $\text{CO}_2$ enrichment

A  $\text{CO}_2$  enrichment experiment was performed outdoors using recirculating cultivation systems at the Marine Research Facility Unit (MARFU) at James Cook University between August and September 2010. Two independent sumps were used, one was supplied directly with  $\text{CO}_2$  gas stream (food grade 99.9% – BOC Australia) and regularly adjusted to maintain pH between 6.5 and 7, whereas the other acted as a control sump with no additional  $\text{CO}_2$ . These systems provided a constant water flow of 2 volumes (vol)  $\text{h}^{-1}$  to polyethylene white buckets with 5 L capacity, 0.035  $\text{m}^2$  surface area, containing a ring of aeration in the bottom to maintain the algae in tumble culture. The buckets were stocked with 3 g fresh weight  $\text{L}^{-1}$  ( $n = 3$  for each species\* $\text{CO}_2$  treatment).

Cultures were acclimated for two weeks at these conditions and a formal growth experiment conducted over the subsequent four week period. Algal biomass of each tank was harvested weekly to determine productivity and subsequently restocked at the original

density of 3 g fresh weight  $L^{-1}$ . The algae were collected in mesh bags (0.1 mm mesh) and the biomass drained to a constant fresh weight in a washing machine (spin cycle 1000 rpm). Productivity ( $g\ m^{-2}\ day^{-1}$  dry weight) was then calculated using equation (2):

$$P = \{[(B_f - B_i)/FW : DW]/A\}/T \quad (2)$$

where  $B_i$  is the initial biomass,  $B_f$  is the final biomass, FW:DW is the fresh to dry weight ratio, A is area of culture vessels and T the number of days in culture. The dry weights were acquired individually for each week from excess centrifuged biomass oven dried at  $65^\circ C$  for 48 h. Resulting FW:DW ratios were on average 3.5:1 for *C. coelothrix*, 5:1 for *C. patentiramea* and 5.9:1 *C. linum*.

The water pH, temperature and salinity were measured daily in the inflow and outflow water of seaweed cultures at 08:00, 12:00 and 18:00 using an YSI 63 multi-parameter meter. Throughout the experiments water temperature and salinity averaged  $28^\circ C$  ( $2 \pm SD$ ) and 35‰ ( $1 \pm SD$ ), respectively. Ambient surface photosynthetic active radiation (PAR) was measured continuously using a LI-192S (2p) sensor placed near the tanks. Daily average PAR recorded during light hours for the experimental period was  $881 \pm 152\ \mu mol\ photons\ m^{-2}\ s^{-1}$ . Water samples were collected twice a week at 12:00 from the inflow and outflow of tumble cultures for alkalinity determination. The samples were fixed with 200  $\mu M$  of saturated  $HgCl_2$  solution, immediately taken to the lab and stored in the fridge until alkalinity analysis. Alkalinity was calculated using potentiometric titration by the Australian Centre for Tropical Freshwater Research (ACTFR) at James Cook University.  $C_i$  concentration and sources were calculated using the pH, alkalinity, salinity, and temperature original values of collection time, using the software  $CO_2sys$  [34]. Nitrogen and phosphorus were measured from water samples collected from the inflow and immediately analysed by cadmium reduction and ascorbic acid techniques (HACH model DR/890), respectively. Average nitrogen and phosphorus concentrations during the experiment were  $\sim 2.4$  and  $0.16\ mg\ L^{-1}$ , respectively.

### Biomass elemental analysis

The biomass of each tank was harvested at the completion of the four week growth period for elemental analysis ( $n = 3$  for each  $CO_2$ \*species treatment). Biomass was spun dry and then oven dried at  $60^\circ C$  for 48 h, milled and stored in glass containers prior to analysis. Nitrogen and carbon were quantified for each sample using isotope analysis. Ash was quantified using a Carlo-Erba elemental autoanalyzer (Environmental Biology Group, Australian National University, Canberra).

### Statistical analysis

Two-way fixed-effect analyses of variance (ANOVA) were used to compare the growth response of the three green tide algal species to different pH and correspondent  $CO_2$  and  $HCO_3^-$  ratios, and the effects of  $CO_2$  enrichment on biomass productivity using the software SYSTAT 12. Post-hoc comparisons were made to assess the differences between treatments in both experiments (Tukey's HSD multiple comparisons). The ANOVA assumptions of homogeneity of variance and normality were assessed by scatter plots and normal curve of the residuals, respectively [35]. To test whether the elemental composition of biomass was influenced by  $CO_2$  enrichment, we used a two-factor permutational multivariate analysis of variance (PERMANOVA, PRIMER v.6). The two fixed-factors were species and  $CO_2$  enrichment, while the dependent variables were the % nitrogen, carbon and ash content

of dried biomass. Biomass composition data was fourth-root transformed for the PERMANOVA.

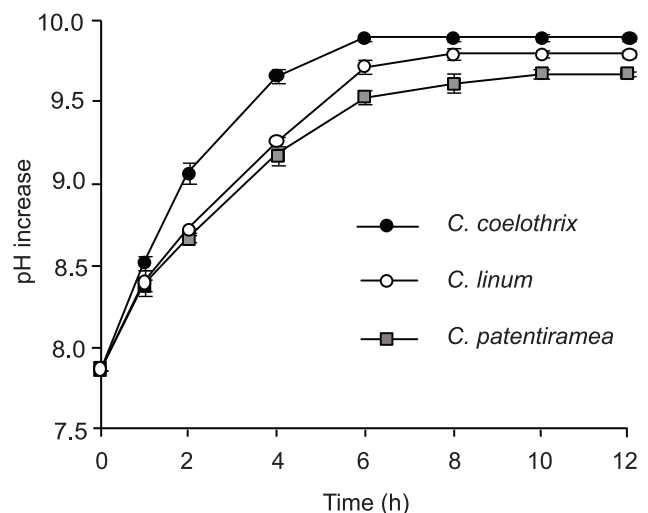
## Results

### pH drift in closed vessel

The pH drifted from 7.9 to over 9.7 for all three algal species (Fig. 1). *C. coelothrix* had the highest pH compensation point of 9.9, which was reached after six h in culture. *C. linum* and *C. patentiramea* used the  $HCO_3^-$  in the water at a slower rate, taking eight and ten hours to achieve the slightly lower pH compensation points of 9.8 and 9.7, respectively (Fig. 1). The relatively faster rate of pH increase for *C. coelothrix* supports more efficient  $HCO_3^-$  use than either *C. linum* and *C. patentiramea*.

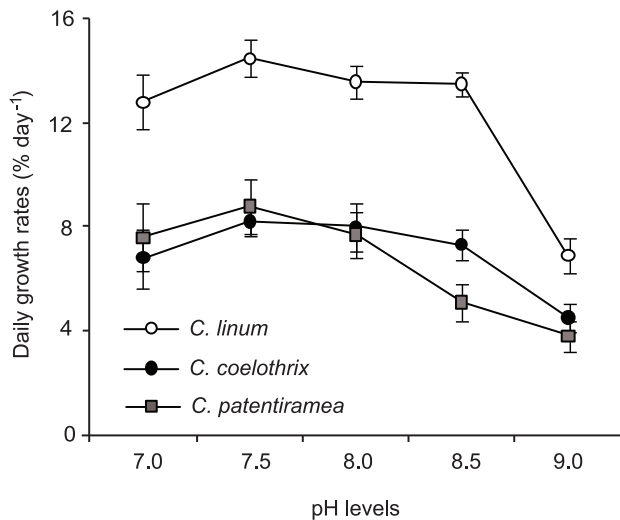
### Effects of pH on algal growth

All three species had decreasing growth rates with increasing pH above the optimum of pH 7.5. However, there was a significant interaction between the species and the pH levels in which they were cultured ( $P < 0.001$ , Table 1, Fig. 2), driven by different optimal pH ranges for growth. In other terms, integrating the pH drift results in the previous section, different growth responses were reflective of different  $HCO_3^-$  affinities. Both *C. coelothrix* and *C. linum* had higher growth rates at pH levels between 7 and 8.5, whereas the optimum pH range for *C. patentiramea* was between 7 and 8 (Fig. 2). There were no significant differences in growth rates within the optimal pH range for each species (Tukey's HSD,  $P > 0.05$ ). The highest individual growth rates for all three species were measured at pH 7.5, with growth rates of 14.5, 8.8 and 8.2%  $day^{-1}$  for *C. linum*, *C. patentiramea* and *C. coelothrix*, respectively (Fig. 2). Growth rates for *C. linum* and *C. coelothrix* decreased above the optimal pH range (from pH 8.5 to pH 9) by 48% and 35% relative to the control, respectively. Growth rates for *C. patentiramea* decreased by 30% relative to the control above the optimal pH range (from pH 8 to pH 8.5). Growth rate further decreased to 47% of the control at pH 9. The highest susceptibility of *C. patentiramea* growth to increasing pH levels (lower  $CO_2:HCO_3^-$  ratio) supports a relatively lower capability of using  $HCO_3^-$ . In accordance with the pH drift experiment, the lower



**Figure 1. pH drift experiment for *C. coelothrix*, *C. patentiramea* and *C. linum*.** Data show mean pH value ( $\pm 1$  SD) for each sampling time ( $n = 3$ ).

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**Figure 2. Growth of *C. coelothrix*, *C. patentiramea* and *C. linum* cultured in different pH levels.** Data show mean daily growth rates ( $\pm 1$  SE) for each pH levels\*species (n = 3). doi:10.1371/journal.pone.0081164.g002

sensitivity of *C. coelothrix* to changes in pH supports its more efficient use of  $\text{HCO}_3^-$ .

### Algal productivity under $\text{CO}_2$ enrichment

Based on the differences in  $\text{HCO}_3^-$  utilization efficiencies between the three species, the subsequent step was to quantify increases in productivity through the addition of  $\text{CO}_2$  in controlled intensive cultures. The addition of  $\text{CO}_2$  decreased the pH of the inflowing seawater from  $\sim$  pH 8 (control) to pH 6.7. Under these conditions the concentration of  $\text{CO}_2$  was twenty five times higher in the  $\text{CO}_2$  enriched cultures compared to the control (Table 2). The addition of  $\text{CO}_2$  also increased the concentration of  $\text{HCO}_3^-$  in the  $\text{CO}_2$  enriched cultures to 2 mM, compared to 1.4 mM in the control cultures, because the hydration of  $\text{CO}_2$  produces carbonic acid, and its subsequent de-protonation leads to the formation of  $\text{HCO}_3^-$ . After passing through the seaweed tanks, at 2 vol  $\text{h}^{-1}$ , all  $\text{CO}_2$  was depleted from water within the control cultures. In contrast, there was a continual supply of  $\text{CO}_2$  in the  $\text{CO}_2$  enriched cultures for photosynthesis.  $\text{HCO}_3^-$  concentration in the control and  $\text{CO}_2$  enriched cultures was  $\sim$ 1 mM and 1.5 mM, respectively. *C. coelothrix* cultures had the lowest concentration of all carbon forms (Table 2), and therefore the highest carbon uptake rates of all three species. *C. patentiramea*

**Table 1. Summary output for significant interactions of the ANOVA and PERMANOVA analyses.**

Source	df	MS	F	P
<b>ANOVA</b>				
Species*pH	12	16.16	7.01	<0.001
Species* $\text{CO}_2$	2	5.91	3.73	0.031
<b>PERMANOVA</b>				
Species* $\text{CO}_2$	2	7.06	16.33	<0.001

ANOVA testing the effects of varying pH on growth and  $\text{CO}_2$  enrichment on algal productivity, and PERMANOVA (Species\* $\text{CO}_2$ ) testing effects of  $\text{CO}_2$  enrichment on biomass elemental composition. doi:10.1371/journal.pone.0081164.t001

**Table 2. Values for pH, dissolved inorganic carbon (Ci), carbon dioxide ( $\text{CO}_2$ ) and bicarbonate ( $\text{HCO}_3^-$ ) for the  $\text{CO}_2$  enrichment experiments.**

	$\text{CO}_2$ enrichment	pH	Ci (mM)	$\text{CO}_2$ ( $\mu\text{M}$ )	$\text{HCO}_3^-$ ( $\mu\text{M}$ )
Inflow	+ $\text{CO}_2$	6.73 $\pm$ 0.26	2.33 $\pm$ 0.20	250 $\pm$ 60	1980 $\pm$ 160
	Control	7.98 $\pm$ 0.16	1.63 $\pm$ 0.12	10 $\pm$ 05	1400 $\pm$ 100
<i>C. coelothrix</i>	+ $\text{CO}_2$	7.42 $\pm$ 0.20	1.60 $\pm$ 0.15	40 $\pm$ 10	1510 $\pm$ 140
	Control	8.52 $\pm$ 0.12	1.17 $\pm$ 0.13	0	930 $\pm$ 130
<i>C. linum</i>	+ $\text{CO}_2$	7.38 $\pm$ 0.19	1.62 $\pm$ 0.14	50 $\pm$ 10	1520 $\pm$ 110
	Control	8.47 $\pm$ 0.10	1.20 $\pm$ 0.13	0	980 $\pm$ 120
<i>C. patentiramea</i>	+ $\text{CO}_2$	7.35 $\pm$ 0.20	1.62 $\pm$ 0.11	50 $\pm$ 10	1525 $\pm$ 100
	Control	8.38 $\pm$ 0.13	1.26 $\pm$ 0.14	0	1100 $\pm$ 140

Data show mean values ( $\pm 1$  SD) from the inflow and outflow of the green tide algal cultures with additional  $\text{CO}_2$  and control (n = 8).

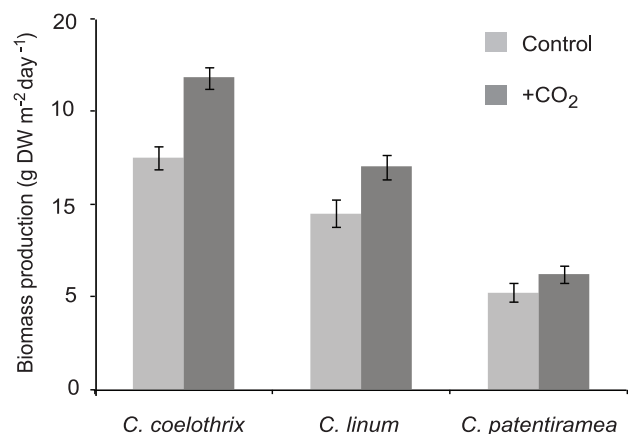
doi:10.1371/journal.pone.0081164.t002

cultures had the highest Ci concentration in the water, in particular in the control treatment, and therefore carbon uptake rates were lower for *C. patentiramea* when only  $\text{HCO}_3^-$  was present. These results confirm the laboratory data indicating that *C. patentiramea* has the least effective  $\text{HCO}_3^-$  utilisation of the three species.

The three algal species had different productivity (growth) responses to  $\text{CO}_2$  enrichment, with a significant interaction between  $\text{CO}_2$  supply and the species tested ( $P < 0.001$ , Table 1). The relative productivity of *C. coelothrix* and *C. linum* were significantly enhanced ( $\sim 26$  and  $24\%$ , respectively) when supplied with additional  $\text{CO}_2$  (Fig. 3). The productivity of *C. coelothrix* increased from 12.5 to 16.8  $\text{g DW m}^{-2} \text{ day}^{-1}$ , and *C. linum* from 9.5 to 12  $\text{g DW m}^{-2} \text{ day}^{-1}$  (Fig. 3). The productivity of *C. patentiramea* (5.2 to 6.2  $\text{g DW m}^{-2} \text{ day}^{-1}$ ) to  $\text{CO}_2$  enrichment was not significantly different to that of the control (Tukey's HSD,  $P > 0.05$ ).

### Biomass elemental analysis

In general, *C. coelothrix* and *C. linum* had higher carbon and nitrogen concentrations and lower ash contents than *C. patentiramea*



**Figure 3. Biomass productivity in response to  $\text{CO}_2$  enrichment for *C. coelothrix*, *C. patentiramea* and *C. linum*.** Data show mean biomass productivity ( $\pm 1$  SE) for each  $\text{CO}_2$  level\*species (n = 3). doi:10.1371/journal.pone.0081164.g003

(Table 3). However, CO<sub>2</sub> enrichment influenced the elemental composition of the algal species in different ways (PERMANOVA, Species\*CO<sub>2</sub>, P<0.001, Table 1). This interaction was mainly driven by positive influence of CO<sub>2</sub> enrichment on carbon and nitrogen content in *C. coelothrix* and *C. linum* compared to the negative influence in *C. patentiramea*, which corresponded with an increase in ash content in the latter (Table 3). *C. coelothrix* biomass increased in carbon and nitrogen content by ~2%, while ash decreased by ~1% relative to the control (Table 3). *C. linum* biomass also increased in carbon and nitrogen content compared to the control, but to different degrees (by ~4% and 2%, respectively). In contrast, the biomass of *C. patentiramea* cultured under CO<sub>2</sub> enrichment had a lower carbon (4%) and nitrogen (1%) content and high ash content (7%) compared with the control (Table 3).

## Discussion

This study demonstrates that three green tide algal species, *C. coelothrix*, *C. linum* and *C. patentiramea*, have the ability to use HCO<sub>3</sub><sup>-</sup> as a complementary carbon source to CO<sub>2</sub> for photosynthesis. However, this ability is restricted to a narrower pH range than for many other green algae belonging to the same genera. There is a lower comparative complexity or efficiency of the mechanisms involved in the uptake or conversion of HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub> for these species. Consequently, this corresponds to a relatively higher dependence on CO<sub>2</sub> as a carbon source for photosynthesis and this is reflected in the significant enhancement of productivity of these species when enriched with CO<sub>2</sub> in intensive culture.

### Algal affinity for HCO<sub>3</sub><sup>-</sup> pH drift in closed vessel

Comparatively, green algae as a taxonomic group photosynthesise at the highest pH levels with compensation points up to pH 10.8 [32]. At these pH levels, CO<sub>2</sub> is absent and HCO<sub>3</sub><sup>-</sup> is the only functional form of inorganic carbon, representing less than a quarter of the total Ci. Active photosynthesis at these pH levels is only possible because of diverse and highly efficient mechanisms to overcome CO<sub>2</sub> constraints through the utilization of HCO<sub>3</sub><sup>-</sup> [36]. There are at least two mechanisms to utilize HCO<sub>3</sub><sup>-</sup> in green macroalgae [37]. The first is the extracellular dehydration of HCO<sub>3</sub><sup>-</sup> into CO<sub>2</sub> through the periplasmic carbonic anhydrase (CA) enzyme, followed by diffusion of CO<sub>2</sub> into the cell, and this is the most widely distributed mechanism. The second mechanism is the direct uptake of HCO<sub>3</sub><sup>-</sup> through the plasma membrane, mediated by an anion exchange protein [38]. Some species of

*Cladophora* have a third mechanism with the uptake of Ci through a vanadate-sensitive P-type H<sup>+</sup>-ATPase (proton pump) [39]. Species with these mechanisms raise the pH up to 10.5 in a closed vessel. The three species in this study did not raise the pH above 9.9 and therefore have limited HCO<sub>3</sub><sup>-</sup> transport. They almost certainly concentrate carbon using the dehydration of HCO<sub>3</sub><sup>-</sup> by CA into CO<sub>2</sub>, as this is the most common mechanism of HCO<sub>3</sub><sup>-</sup> utilization in algae [40]. This mechanism usually operates at ~pH 8.3, when the proportion of CO<sub>2</sub> in the total Ci pool is below 1% and HCO<sub>3</sub><sup>-</sup> is more than 90%. The capacity to utilize HCO<sub>3</sub><sup>-</sup> through this mechanism decreases sharply with increased pH, and is ineffective at pH 9.8 [41]. The direct transport of HCO<sub>3</sub><sup>-</sup> through an anion exchange protein usually operates at higher pH (~9.3) [39], and is the most probable mechanism for compensation points above 9.5 in the three species. These two mechanisms operate separately in other species of green algae with periplasmic CA activity dominating at lower pH, and direct uptake of HCO<sub>3</sub><sup>-</sup> by an anion exchanger at higher pH [39]. The incapacity of the species in this study to raise the pH above 9.7–9.9 suggests that there is no proton pump mechanism involved in HCO<sub>3</sub><sup>-</sup> transport. [42] inhibited the proton pump mechanism in *Ulva procera*, an alga capable of a pH compensation point of 10.5, and the pH remained below 9.9, demonstrating a reliance on this third mechanism to elevate the pH compensation to its highest level.

In a comparative context, the two *Cladophora* species in this study are less efficient in the use of HCO<sub>3</sub><sup>-</sup> than other species from the same genera with pH compensation points of ~pH 10.5 [32,42]. However, as in this study, some species of *Cladophora* maintain a preference for dissolved CO<sub>2</sub> as a carbon source [43]. These different responses may be related to the environmental niche prior to experiments, as the ability of algae to utilise HCO<sub>3</sub><sup>-</sup> is strongly related to habitat [32]. Individuals of the same species can express alternate strategies for carbon acquisition when in different habitats, or the habitat itself might select for survival of genotypes with different carbon acquisition strategies [31]. This relatively limited ability to utilise HCO<sub>3</sub><sup>-</sup> is reflected in the growth response of all three species at different pH environments in this study.

### Effects of pH on algal growth

As pH increases from 7 to 8, the relative proportion of Ci present as CO<sub>2</sub> is reduced by over 70%, while the relative proportion of HCO<sub>3</sub><sup>-</sup> decreases by only 10%. This drastic change in the CO<sub>2</sub>:HCO<sub>3</sub><sup>-</sup> ratio had no effect on the growth of algae in this study. The comparative ratio of CO<sub>2</sub>:HCO<sub>3</sub><sup>-</sup> was maintained at each pH throughout the experiment through the addition of a biological buffer. Consequently, CO<sub>2</sub> is always available between pH 7 and 8 at concentrations that meet the carbon requirement for algal photosynthesis and growth. This does not, however, exclude the activation of the CA mechanism at ~pH 8, which supplies additional CO<sub>2</sub> derived from HCO<sub>3</sub><sup>-</sup> to compensate for its lower availability at increased pH [19].

From pH 8 to 8.5, CO<sub>2</sub> decreases markedly and photosynthesis and growth depends on the efficiency of HCO<sub>3</sub><sup>-</sup> utilization mechanisms. The growth rates of *C. linum* and *C. coelothrix* within this pH range changed little. In contrast, a significant decrease in growth for *C. patentiramea* confirms that it is the least adapted to grow in the absence of CO<sub>2</sub>. Above pH 8.5, HCO<sub>3</sub><sup>-</sup> is replaced by CO<sub>3</sub><sup>2-</sup> and growth decreased significantly for all species. Steeper decreases in growth rates for *C. patentiramea* and *C. linum* between pH 8 and 9, compared to *C. coelothrix*, correspond with the slower rate of increasing pH for these two species in the pH drift experiment. These data, together with the highest pH compensation point, confirm that *C. coelothrix* has the most efficient

**Table 3.** Values for % carbon (C), % nitrogen (N) and % ash from algal biomass cultured with CO<sub>2</sub> enrichment and control.

Species	CO <sub>2</sub> enrichment	% C	% N	% Ash
<i>C. coelothrix</i>	+CO <sub>2</sub>	33.29±0.28	6.05±0.03	25.20±0.55
	Control	30.97±0.30	4.07±0.03	26.22±0.56
<i>C. linum</i>	+CO <sub>2</sub>	31.67±0.70	5.98±0.10	29.20±0.88
	Control	27.12±0.13	4.12±0.13	33.43±0.47
<i>C. patentiramea</i>	+CO <sub>2</sub>	18.05±0.45	3.07±0.08	56.97±1.97
	Control	22.53±1.06	4.27±0.17	50.66±1.53

Data show mean values (±1 SD) of % dried biomass for each species\* CO<sub>2</sub> treatment (n=3).

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mechanisms of  $\text{HCO}_3^-$  utilisation. However, in a broader comparative context, the relatively low pH compensation points and significant decreases in growth rates from pH 8 to 9, again demonstrate that the three species in this study are not as efficient in the use of  $\text{HCO}_3^-$  as a carbon source compared to many other green tide algal species.

### Algal productivity under $\text{CO}_2$ enrichment

The productivity of two of the three species of green tide algae, *C. coelothrix* and *C. linum*, was enhanced through the addition of  $\text{CO}_2$ . Notably, the enrichment treatment had twenty five times more  $\text{CO}_2$  available than the control. This maintained the pH of the enriched water below pH 7.5 (excess  $\text{CO}_2$ ), whereas the pH of the control cultures averaged 8.5 (depleted  $\text{CO}_2$ ). Considering the relatively limited ability of species to utilize the  $\text{HCO}_3^-$  pool, and that this process has an energetic cost [28], the constant presence of  $\text{CO}_2$  at pH 7.5 disproportionately facilitated photosynthetic carbon fixation, and ultimately enhanced biomass productivity. Enhanced growth rates with  $\text{CO}_2$  enrichment have also been reported for other species capable of using bicarbonate [20–23]. However, the magnitude of the growth responses to  $\text{CO}_2$  enrichment is to some extent dependent on the efficiency of carbon concentrating mechanisms for each species. For example, species depending almost exclusively on  $\text{CO}_2$  for photosynthesis can increase their biomass productivity up to three times when cultured in enriched  $\text{CO}_2$  culture media [19,44,45]. Any differences in growth relative to enhanced  $\text{CO}_2$  can also be due to the effect of  $\text{CO}_2$  on the rate of nitrogen assimilation [46,47]. High levels of  $\text{CO}_2$  can increase the rate of nitrogen assimilation in some algae by up-regulating nitrate reductase, the main enzyme in the nitrate assimilatory pathway [47,48]. This may be the case for *C. coelothrix* and *C. linum* in this study where they have a higher nitrogen content under  $\text{CO}_2$  enrichment. Higher nitrogen and carbon contents on top of increased productivities with  $\text{CO}_2$  enrichment represents a clear advantage for integrated systems focused on biomass production for bioremediation of waste streams [14,49].

In contrast, the nitrogen content of *C. patentiramea* decreased under  $\text{CO}_2$  enrichment, suggesting no effect on assimilation. The effect of high  $\text{CO}_2$  on the assimilation of nitrogen in algae is not consistent with decreases in assimilation for other species of algae

[24,50]. This effect may contribute to the relatively lack of increase in productivity of *C. patentiramea* under  $\text{CO}_2$  enrichment. Notably, laboratory experiments suggested that *C. patentiramea* should be the most sensitive species to  $\text{CO}_2$  enrichment based on relative capabilities of  $\text{HCO}_3^-$  utilization, which indicates that controlled, static, laboratory experiments may not be efficient to predict responses in flow environments (e.g. similar to commercial scale), potentially because of boundary layer/water motion effects on  $\text{Ci}$  distribution [51]. An alternative but related driver to water motion is the morphological differences between the green tide algae. The two rapidly growing species which had enhanced growth under  $\text{CO}_2$  enrichment, *C. coelothrix* and *C. linum*, have a fine filamentous morphology suitable for tumble culture. In contrast, *C. patentiramea* has tightly interwoven filaments (e.g. ball-like structure) that restrict light to the inner filaments (auto-shading), thereby potentially limiting photosynthesis and growth. These physical factors may have influenced small density cultures in the laboratory in a different way than the dense cultures in the outdoor experiment, where individual, larger clumps could become limited. Regardless, *C. patentiramea* is not a good option for intensive cultivation because despite the lack of growth response to additional  $\text{CO}_2$ , high  $\text{CO}_2$  affected negatively the nitrogen and carbon content while increasing ash content, and therefore the amount of biomass that can be converted into soil conditioners [52] or biofuels [9] decreases substantially.

In conclusion, intensive cultures of *C. coelothrix* and *C. linum* enriched with  $\text{CO}_2$  had significantly enhanced productivity, despite their ability to utilise  $\text{HCO}_3^-$ . This demonstrates the potential for enhanced production for these species using  $\text{CO}_2$  enrichment. This can be integrated with the industrial production of  $\text{CO}_2$  and waste water streams from industry [53] to deliver a model where algae provide a bioremediation service of both air ( $\text{CO}_2$ ) and water (nitrogen, phosphorous, metals and trace elements), and an opportunity to utilise this biomass for bio-energy products.

### Author Contributions

Conceived and designed the experiments: PPS RdN NP LM. Performed the experiments: PPS. Analyzed the data: PPS NP LM. Wrote the paper: PPS RdN NP LM.

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