

# Cultivation of *Chondrus crispus* in a closed system bubble column photobioreactor

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## Abstract

A closed system bubble column photobioreactors usually applied in microalgae studies and productions was tested on batch cultures of *Chondrus crispus* (Rhodophyta). Carbon dioxide was added to maintain constant pH. Two types of inocula were cultivated to compare specific growth rate, degree of epiphyte growth and biomass composition. In the first experiment inoculum collected from a natural population in Denmark was used. Inoculum in the second experiment was prepared from the newly formed and visibly clean thalli of biomass obtained from the first experiment. The specific growth rates of *C. crispus* were determined from the slope of the rectilinear part of a curve of  $\ln(dx/dt)$  as function of time using on-line carbon dioxide addition as estimate of biomass  $x$ . With this method, the first experiment showed three exponential growth periods with specific growth rates of  $0.047\text{ d}^{-1}$ ,  $0.48\text{ d}^{-1}$ , and  $0.059\text{ d}^{-1}$ , whereas the second experiment showed only one specific growth rate of  $0.12\text{ d}^{-1}$ . The specific growth rates were furthermore determined from the commonly used calculations with biomass increase assuming exponential growth throughout the entire culture period and were  $0.066\text{ d}^{-1}$  and  $0.095\text{ d}^{-1}$  in the first and second experiment, respectively. These experiments show that the closed system bubble photobioreactor designed for microalgae growth can be used for *C. crispus* growth, giving on-line information of growth and indicating shifts in specific growth rates during culture period, not recognised in commonly used calculations. In addition, this method indicates that fragments of fresh thalli as inoculum reduce growth of epiphytes.

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Key words: Batch culture, carbon addition, C:N ratio; specific growth rate

## 1. Introduction

Cultivation under controlled conditions in photobioreactors provides information about the effects of physiochemical variables on the algal physiology. Previously macroalgae cultures in photobioreactors have only been investigated in so-called suspension cultures, which consisted of callus produced from *Saccharina latissima* (Qi and Rorrer, 1995; Rorrer et al., 1995), *Agardhiella subulata* (Huang and Rorrer, 2002a,b), *Acrosiphonia coalita* (Rorrer et al., 1996), *Laminaria japonica* (Gao et al., 2005; Gao et al., 2006), *Undaria pinnatifida* (Xu et al., 2002) and *Kappaphycus alvarezii* (Munoz et al., 2006). Both stirred tank-, airlift- and bubble column photobioreactors were used for cultivation of these macroalgae, but on-line computer control and on-line data acquisition were limited. The closed system bubble column photobioreactor with on-line optimization of light intensity and regulation of pH, temperature and dissolved oxygen tension is designed to provide detailed information about microalgal growth. On-line measurements obtained in microalgae investigations show that carbon dioxide addition needed to maintain constant pH reflects metabolic activity and can therefore be used to estimate specific growth rates (Poulsen and Iversen, 1999; Christensen, 2007). The slope of the rectilinear part of a curve of  $\ln(dx/dt)$  using the carbon dioxide addition as estimate of biomass concentration,  $x$  as function of time is equal to the specific growth rate (Poulsen et al., 2003).

A major problem in macroalgal photobioreactor cultivation is elimination of epiphytes and contaminants such as bacteria. In order to achieve axenic suspension cultures, techniques of callus induction is used to harvest filamentous parts, which are used as inoculum for photobioreactor cultivation (Qi and Rorrer, 1995; Rorrer et al., 1995; Huang and Rorrer, 2002a,b; Rorrer et al., 1996; Gao et al., 2005; Gao et al., 2006; Xu et al., 2002; Munoz et al., 2006), or generated the axenic callus from explants (Huang and Fulita, 1997). Another strategy is to treat the algae with chemicals and antibiotic to eliminate growth of diatoms, other microalgae, bacteria and epiphytes (Garcia-Jimenez et al. 1999; Choi et al., 2002). A third strategy is to favour growth of the red algae by enhancing the macroalgal density and thereby reducing the light penetration eliminating growth of epiphytes (Lüning and Pang, 2003; Bidwell et al, 1985). Finally, it is possible to reduce the amount of nutrients making survival of fast growing microalgae or

ephemeral macroalgae minimal as perennial macroalgae are capable of surviving low nutrient availability of longer periods (Bidwell et al., 1985; Pedersen and Borum, 1996).

In this study we tested the application of the closed system bubble column photobioreactor to provide detailed information on growth of the red macroalgae *Chondrus crispus* Stackhouse. The extracted on-line data are validated and compared to conventional data analysis. *Chondrus crispus* was chosen for these experiments because it is a major source of high value carrageenan, which is used in pharmaceuticals or gelling agent in e.g. foods, diary products, and tooth paste (Bixler, 1996; Carte, 1996).

## 2. Materials and methods

### 2.1. Photobioreactor and regulations

A 37 L bubble column photobioreactor with a working volume of 33 L designed for cultivation of microalgae (Christensen, 2007) was modified by placing a polycarbonate grid 0.40 m above the bottom to keep the macroalgae in a homogenous light regime and to avoid entrapment of thalli below the spargers and other insertions (Figure 1).

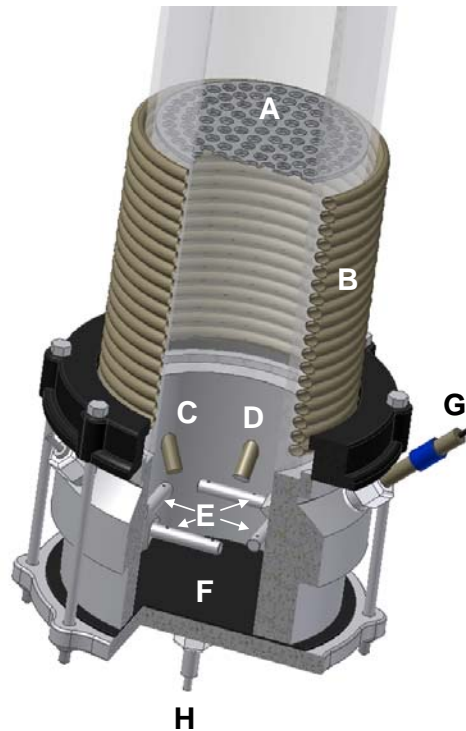


Figure 1. Bottom part of the bubble column photobioreactor. A: Polycarbonate grid, B: Cooling tube, C: Temperature pocket, D: Oxygen electrode, E: Ring sparger, F: Membrane diffuser, G: pH electrode and H: Inlet to membrane diffuser

A closed system was created by pumping the effluent gas back into the photobioreactor through a membrane diffuser ( $1 \text{ L min}^{-1}$ ) to generate small bubbles for efficient mass transfer and a ring sparger ( $2.5 \text{ L min}^{-1}$ ), which generates larger bubbles providing mixing (Poulsen and Iversen, 1999). Photosynthetically produced oxygen was removed and quantified by addition of hydrogen over palladium catalyst (Delente et al., 1991; Eriksen et al, 2007) thereby maintaining constant dissolved oxygen tension. Constant pH at 8.0 was achieved by addition of carbon dioxide. All additions were registered on-line every 60 seconds by a computer. Photosynthetic quotient defined as  $\Delta\text{O}_2, \text{ produced} / \Delta\text{CO}_2, \text{ assimilated}$

was estimated from the slope of the curve showing the correlation between oxygen evolution and carbon dioxide addition. Continuous light was supplied from a cabinet consisting of 14 fluorescent tubes (58 W) surrounding the photobioreactor and the irradiance was regulated between 20-200  $\mu\text{E m}^{-2} \text{s}^{-1}$  in order to optimize the carbon dioxide addition rate (Eriksen et al., 1996; Eriksen et al., 2007).

Temperature was regulated at 20 °C by cooling of water and with cooling tube around the bottom part of the photobioreactor surface (Figure 1).

### 2.2. Determination of characteristic mixing time

Characteristic mixing time was determined by a tracer pulse method to evaluate the effect of the grid. The photobioreactor contained a linear buffer (Poulsen and Iversen, 1997) supplemented with 0.25 M NaCl and was equipped with pH-electrodes at the bottom and top. A tracer pulse of alkali was added at the top of the photobioreactor and the exponential decrease of the pH difference was followed as function of time. The negative inverse of the first order rate constant is the characteristic mixing time.

### 2.3. Stock culture, inoculum and medium

Non-reproductive *Chondrus crispus* was collected at the boulder shore in Gilleleje, Denmark (56°07.5'N, 12°18.3E) on December the 6<sup>th</sup> 2005 and was acclimated from 7 °C to 20 °C stepwise (2 °C increase every 2-3 days). Salinity was increased from 20 to 25 ‰ in two steps in an aerated plastic container containing 30 L f/2 medium (Guillard, 1975). The biomass was maintained in the aerated container under 8:16 (L:D) illumination with irradiance of 30  $\mu\text{E m}^{-2} \text{s}^{-1}$ , which resembles the day length when the biomass was collected. The f/2 medium was changed weekly with addition of germanium dioxide (final concentration of 0.01 g L<sup>-1</sup>) in three of the medium changes in order to eliminate growth of diatoms (Garcia-Jimenez et al., 1999). The biomass was maintained for one month before the first photobioreactor experiment. f/2 medium with a salinity of 25 ‰ was used for the batch cultivations performed in bubble column photobioreactor. The f/2 nutrient source is standard growth medium for mass cultivation of *C. crispus* (pers. comm. Hafting, Acadian Seaplant, 2008).

In the first experiment thalli consisting of stipe and frond were selected from size class II and III (3-7 cm) (Taylor and Chen, 1973) and used as inoculum. The resorcinol test for life history phases of Gigartinae (Garbary and DeWreede, 1988) was used to determine the percentage of gametophytes and tetrasporophytes in the stock culture. The test showed that 90 % were gametophytes.

The inoculum of the second experiment was prepared as fragments of freshly formed thalli from the first experiment (Figure 2). These fragments were stored for three weeks in an aerated beaker (2 L) with f/2 medium under continuous illumination with irradiance of  $100 \mu\text{E m}^{-2} \text{s}^{-1}$ . The medium in the beaker was changed weekly.



Figure 2. *Chondrus crispus* thallus from the end of the first experiment in the closed system bubble column photobioreactor. White lines represent suitable cutting lines for preparation of inoculum for the second experiment.

In both experiments 11 g of blotted wet weight was used as inoculum comprising 78 thalli and 42 fragments of thalli in the first and second experiment, respectively. Prior to inoculation epiphytes were removed manually and the biomass was treated with sodium hypochlorite ( $0.3 \text{ g L}^{-1}$ ) for 2 minutes to avoid growth of microalgae, fungi, bacteria and macro-epiphytes and washed thoroughly with autoclaved seawater followed by treatment with antibiotic ( $0.11 \text{ g L}^{-1}$  Meronem, AstraZeneca, UK) in f/2 medium overnight to avoid bacterial growth (Garcia-Jimenez et al., 1999).

In order to evaluate specific growth rates of individual thalli of *C. crispus*, 9 and 10 replicates were tagged with colored plastic beads in the first and second experiment, respectively (Hami midi-bead: length 5 mm, diameter 5 mm) tied with nylon fishing line (0.35 mm).

#### 2.4. Biomass and substrate analyses

The blotted wet weight (WW) and dry weight (DW) of thalli was found gravimetrically by dapping thalli until visibly dry or drying thalli at 110 °C for 24 hours, respectively, and after that dry weight samples were frozen until further analysis.

Tissue samples (n = 5) of inoculum and final biomass were analyzed for specific carbon and nitrogen concentration (% of dry weight) by a CHNS – Automatic Elemental Analyzer (EA 1110 CHNS, CE Instruments, Milan, Italy).

Growth of epiphytes was evaluated weighing the blotted wet *Chondrus crispus* thalli before and after removal of the epiphytes. Additionally suspended epiphytes and contaminants were measured by filtration of medium at the end of the experiments.

Samples of medium were frozen daily until analysis and the concentration of nitrate was measured spectrophotometrically at 220 nm and 275 nm after addition of hydrochloric acid, correcting for absorbance of organic matter by subtracting twice the absorbance at 275 nm from that of 220 nm (Clesceri et al., 1989). Phosphate concentration in medium samples was determined in presence of ascorbic acid, antimony, and sulphuric acid and measured spectrophotometrically at 880 nm (Murphy and Riley, 1962).

#### 2.5. Specific growth rate

Specific growth rates ( $\mu$ ) were calculated using Equation 1, where  $x$  is the final biomass concentration and  $x_0$  is the initial biomass concentration.  $t-t_0$  is the time of growth. Equation 1 was also used calculating specific growth rates for each tagged replicate.

$$\mu = (\ln(x) - \ln(x_0)) / (t - t_0) \quad (\text{Equation 1})$$

Specific growth rates were furthermore estimated from the slope of the rectilinear part of a curve showing  $\ln(dx/dt)$  as function of time (Poulsen et al., 2003). Averages are presented as averages  $\pm$  standard deviation.

### 3. Results

Two batch cultures of *Chondrus crispus* were investigated in a closed system bubble column photobioreactor with different inocula. The bubble column photobioreactor was suitable for cultivation of thalli of macroalgae suspended in the liquid phase. Sparger bubbles provided sufficient mixing to keep the thalli homogeneously distributed throughout the bubble column photobioreactor without causing visible damage to the algae. The insertion of the grid did not change the characteristic mixing time (Figure 3). A membrane diffuser gas flow of  $1 \text{ L min}^{-1}$  and sparger gas flow of  $2.5 \text{ L min}^{-1}$  gave a characteristic mixing time of 35 sec.

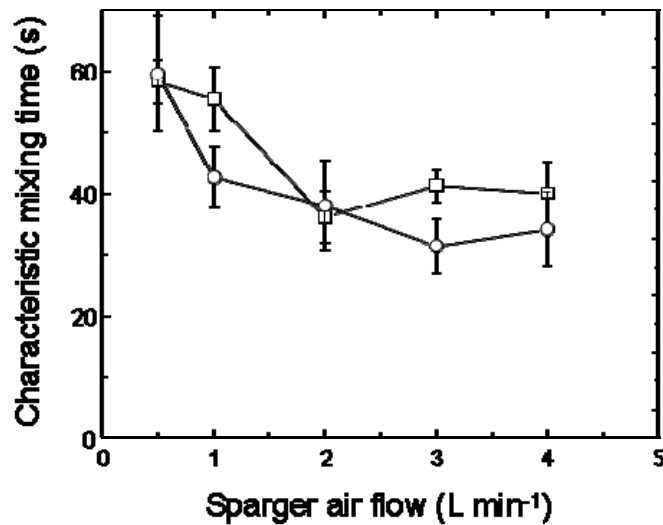


Figure 3. Characteristic mixing time of bubble column photobioreactor used in the experiments: With grid (□). Without grid (○)

The photosynthetic quotient (PQ) of *C. crispus* was 1.0 in both experiments with regression ( $R^2$ ) of 0.9996 and 0.9995 in the first and second experiment, respectively (PQ for the first experiment shown in Figure 4).

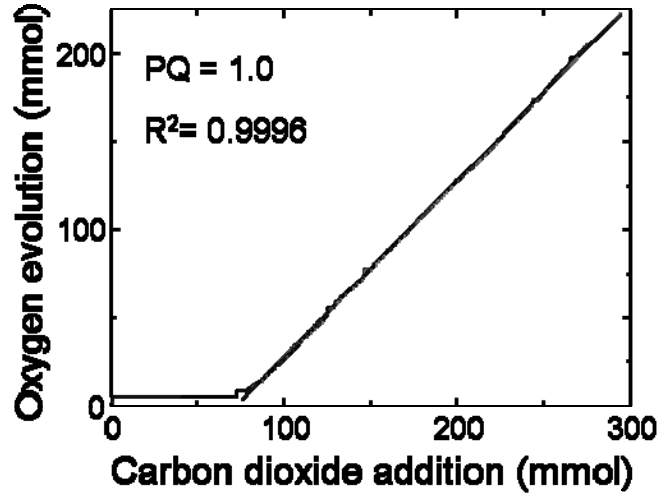


Figure 4. Carbon dioxide added (mmol) in the photobioreactor versus oxygen evolved (mmol)(removed over the palladium catalyst) in the first experiment. This showed a photosynthetic quotient (PQ) of 1.0.

On-line measurements of carbon dioxide addition, irradiance and calculated values of  $\ln(dx/dt)$  and off-line nutrient measurements of the batch culture of *C. crispus* in the first experiment, are shown in Figure 5. The concentration of nitrate and phosphate decreased throughout the batch culture and phosphate was depleted 14 days after inoculation and nitrate was reduced by 91 % within 25 days (Figure 5a). The  $\ln(dx/dt)$  curve indicates a lag-phase of 7 days duration characterized by non-linear relationship of  $\ln(dx/dt)$  as function of time, which is followed by three exponential phases with different specific growth rates;  $0.047 \text{ d}^{-1}$ ,  $0.48 \text{ d}^{-1}$  and  $0.059 \text{ d}^{-1}$ (Figure 5b).

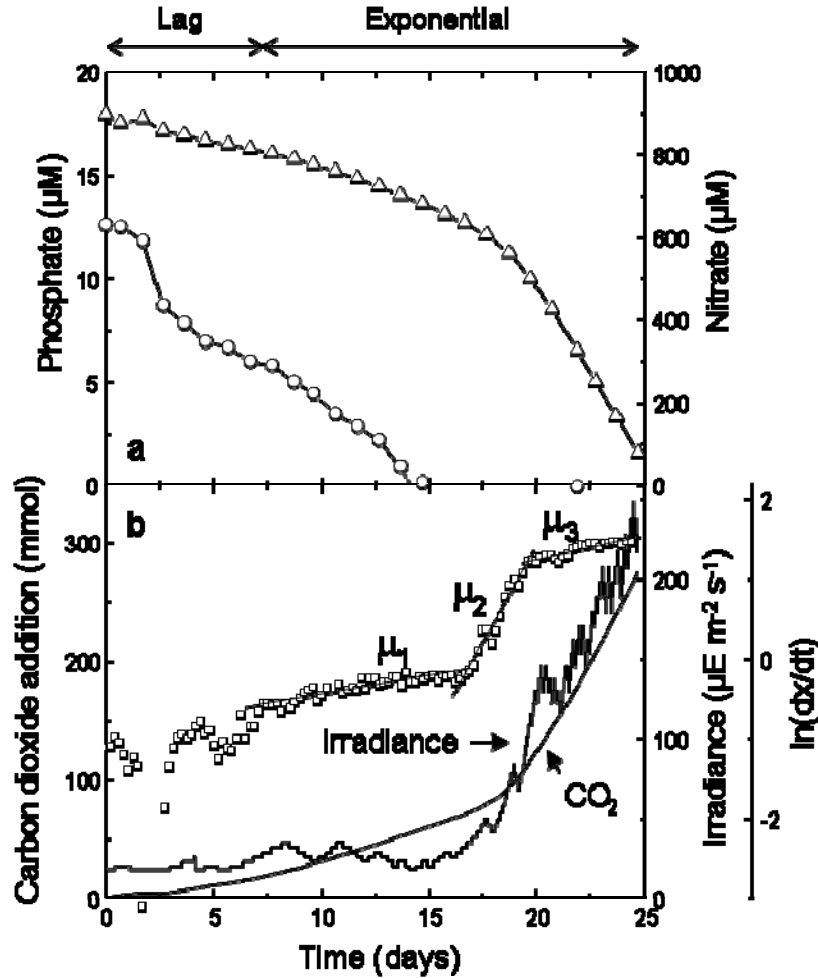


Figure 5. Results of a batch culture of *Chondrus crispus* using inoculum collected from a natural population in the first experiment. (a): Off-line measurements of nitrate ( $\Delta$ ), and phosphate ( $\circ$ ). (b): On-line measurements of carbon dioxide addition, irradiance and calculated values of  $\ln(dx/dt)$  using carbon dioxide addition as estimate of  $x$  ( $\square$ ). Specific growth rates were determined from the slopes of the rectilinear parts,  $\mu_1 = 0.047 \text{ d}^{-1}$ ,  $\mu_2 = 0.48 \text{ d}^{-1}$ , and  $\mu_3 = 0.059 \text{ d}^{-1}$ .

Illumination was automatically regulated to optimize the carbon dioxide addition rate and increased from irradiance of 20 to  $250 \mu\text{E m}^{-2} \text{ s}^{-1}$  (Figure 5b). Growth of tagged thalli was estimated and an example of the same thallus at the beginning of growth and after 25 days of batch cultivation is shown in Figure 6.

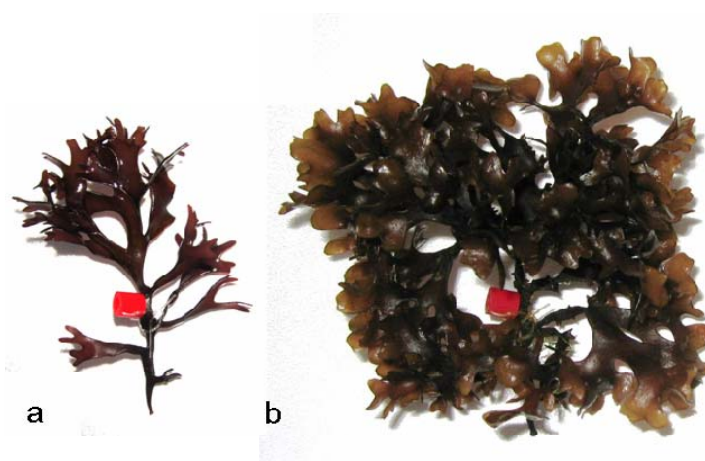


Figure 6. Growth of *Chondrus crispus* in bubble column photobioreactor in the first experiment with inoculum collected from a natural population. Tagged thallus at beginning (a) and end (b) of the 25 days of batch cultivation. Bead is 0.5 cm long.

Phosphate and nitrate were depleted at day 11 and 18, respectively in the second experiment (Figure 7a). Only a short lag-phase (2 days) was identified and exponential growth occurred at a single specific growth rate ( $0.12 \text{ d}^{-1}$ ) determined from the  $\ln(dx/dt)$  curve in Figure 7b. Post-exponential phase was identified from day 16 to 22 (Figure 7b). An example of growth of tagged thallus from the second experiment is shown in Figure 8.

Specific growth rates calculated using Equation 1, were compared to those obtained from the  $\ln(dx/dt)$  vs. time curve.

Table 1. Biomass production as wet weight, specific growth rates ( $\mu$ ) calculated from Equation 1 and  $\ln(dx/dt)$  as function of time, where  $dx/dt$  is numerically derived from the carbon dioxide addition

	Biomass production g	$\mu$ (Eq. 1) $\text{d}^{-1}$	$\ln(dx/dt)$ $\text{d}^{-1}$
Experiment 1	44	0.066	0.047 0.48 0.059
Experiment 2	75	0.095	0.12

In the first experiment the average specific growth rate of tagged thalli was  $0.061 \pm 0.026 \text{ d}^{-1}$ , whereas  $0.098 \pm 0.015 \text{ d}^{-1}$  in the second experiment.

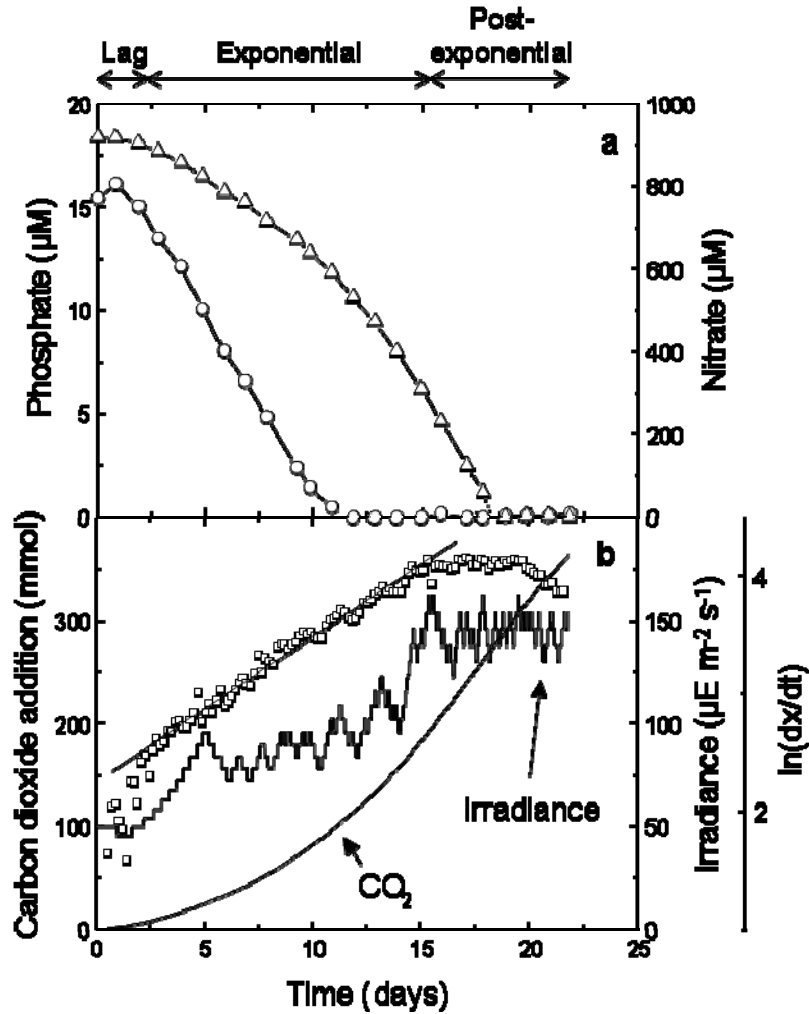


Figure 7. Results of a batch culture of *Chondrus crispus* using inoculum generated from newly formed thalli in the second experiment. (a): Off-line measurements of nitrate ( $\Delta$ ), and phosphate ( $\circ$ ). (b): On-line measurements of carbon dioxide addition, irradiance and calculated values of  $\ln(dx/dt)$  using carbon dioxide addition as estimate of  $x$  ( $\square$ ). Specific growth rate of  $0.12 \text{ d}^{-1}$  were determined from the slopes of the rectilinear parts.

Epiphytes were absent until the 16<sup>th</sup> day in the first experiment, however the wet weight of epiphytes was insignificant compared to the *C. crispus* biomass increase at the end of the experiment. Epiphytes were not observed on the thalli at the end of the second experiment.

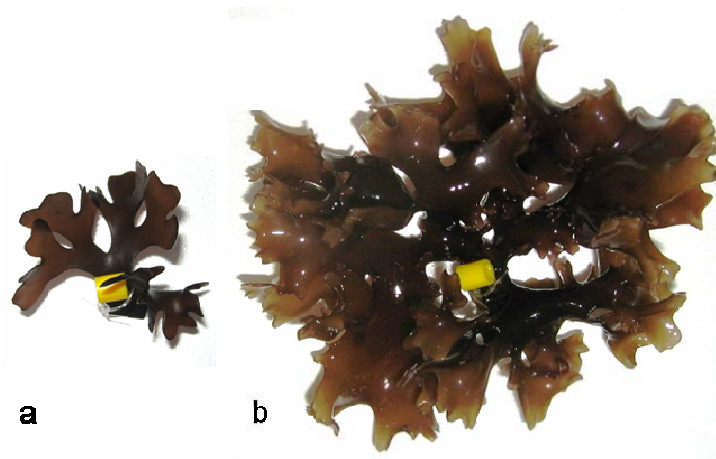


Figure 8. Growth of *Chondrus crispus* in bubble column photobioreactor in the second experiment with tagged thallus at the beginning (a) and end (b) of the 22 days of batch cultivation. Bead is 0.5 cm.

Nitrate removal by the macro algae was in both experiments proportional to carbon dioxide added in the closed system with a slope of 10 and 9 in the first and second experiment, respectively (Figure 9).

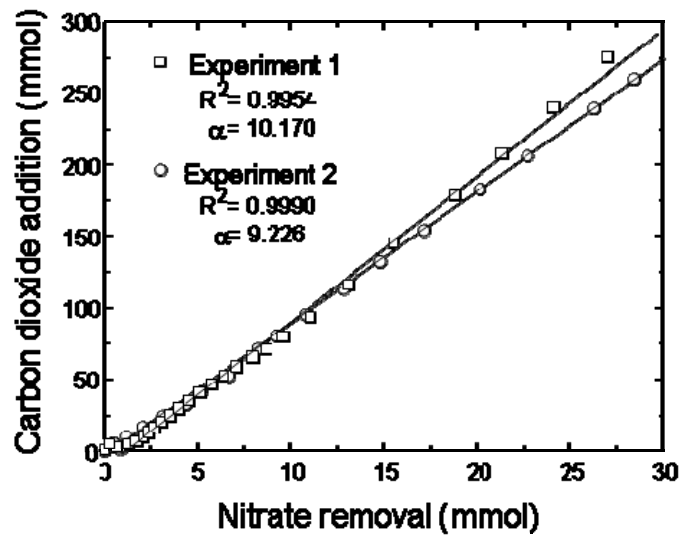


Figure 9. Nitrate removal (mmol) by *Chondrus crispus* in batch cultures the first and second experiment versus carbon addition added (mmol) in the photobioreactor to maintain constant pH, reflecting metabolic activity.

The specific nitrogen concentrations tended to decrease during growth (Table 2).

Table 2: Tissue content of carbon (C) and nitrogen (N) and the ratio between dry weight (DW) and wet weight (WW) of *Chondrus crispus* in the first and second experiment. Data are presented as averages±standard deviation.

	DW:WW ratio		C:N ratio		Tissue content			
					C		N	
	beginning	end	beginning	end	beginning	end	beginning	end
Exp 1	0.26 ± 0.03	0.24 ± 0.02	9	11	30.9 ± 1.98	31.4 ± 1.86	4.0 ± 0.17	3.4 ± 0.29
Exp 2	0.18 ± 0.01	0.15 ± 0.02	11	15	31.2 ± 0.58	33.7 ± 1.72	3.2 ± 0.29	2.6 ± 0.30

The ratio between dry weight and wet weight tended to decrease during growth in both experiments.

Mass balances were performed to estimate the recoveries of carbon and nitrogen in the biomass (Table 3). The recovery of nitrogen was 81 % and 75 % for the first and second experiment, respectively, whereas the carbon balance showed that less than 3 % of the carbon added to the system was not accounted for.

Table 3. Mass balance of nitrogen or carbon.

	Experiment 1	Experiment 2
Nitrogen added (mmol)	29.6	30.3
Biomass nitrogen increase (mmol)	23.9	22.7
Nitrogen recovery (%)	81	75
Carbon added (mmol)	275	363
Biomass carbon increase (mmol)	269	362
Carbon recovery (%)	98	99

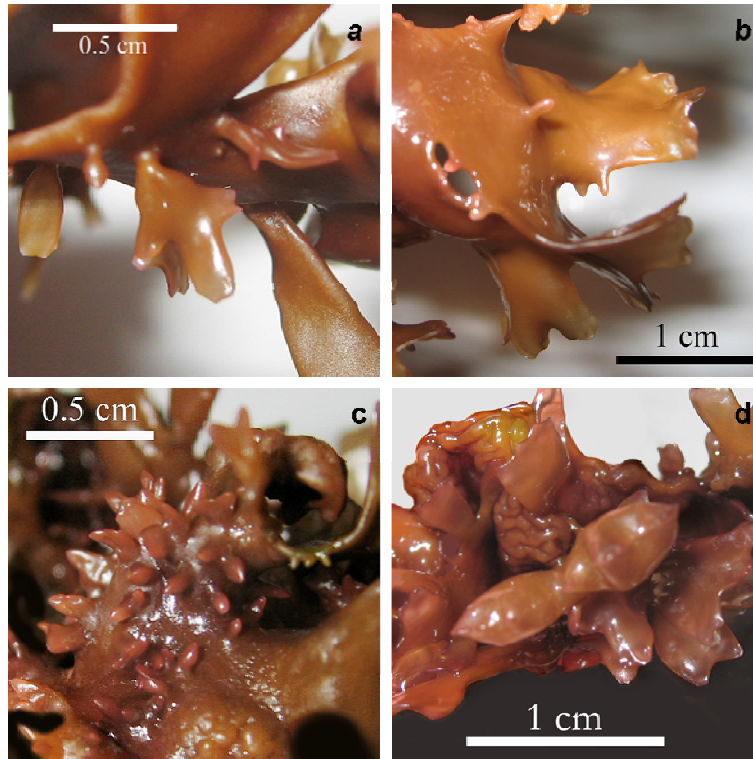


Figure 10. Morphology at the end of the second experiment. (a), (b), and (c): Proliferation from surface of thallus. (d): Bladder with gas in apical thallus.

A few morphological changes were observed in the experiments. In both batch cultures excessive proliferation of marginal bladelet occurred from the stipe and the apical part of thallus, but excessive proliferation from the surface of thalli only occurred in the second experiment (Figure 10a-c). Additionally, bladders filled with gas were observed in the newly formed apical thalli in the second experiment (Figure 10d). Proliferation was never observed from the cut part of the thalli.

#### 4. Discussion

A closed gas system was created, where effluent gas was pumped back into the photobioreactor and this configuration gave more detailed information about growth than would have been possible in open systems. Carbon dioxide addition needed to maintain constant pH was registered on-line. This reflected metabolic activity, also evidenced by a photosynthetic quotient of 1.0. Furthermore, mass balances showed that roughly all of the carbon dioxide added to the system was recovered in the biomass. In addition, the tissue C:N ratio of 11 at end of the first experiment is also found in the on-line carbon dioxide addition correlation with the nitrate removal, giving a slope of 10. However, tissue C:N ratio at the end of the second experiment of 15 is higher than the slope showing 9 mmol carbon dioxide are added for each mmol nitrate removed (Figure 9). In a previous study Atkinson and Smith (1983) related the C:N ratio to the nutritional load and showed that thalli from low nutrient environments have higher tissue C:N ratios. This explains our C:N ratios as nitrogen limitation was absent throughout the first experiment, resulting in low tissue C:N ratio, in comparison to the higher tissue C:N ratio in the second experiment after 4 days of nitrate depletion.

By using carbon addition as a measure of metabolic activity,  $\ln(dx/dt)$  plotted as function of time identifies the exponential growth phase(s). This is seen in the first experiment, as the  $\ln(dx/dt)$  revealed three different specific growth rates of  $0.047 \text{ d}^{-1}$ ,  $0.48 \text{ d}^{-1}$  and  $0.059 \text{ d}^{-1}$ , whereas the second experiment only showed a single specific growth rate of  $0.12 \text{ d}^{-1}$ . The changes in specific growth rate throughout a batch culture most likely reflect the quality difference of the inoculum, i.e. the undefined inoculum in the first experiment as the age of the collected thalli was unknown. In contrast, only a single exponential growth phase was observed when fresh thalli with a well defined history were used as inoculum in the second experiment.

The specific growth rate determined from the  $\ln(dx/dt)$  vs. time curve ( $0.12 \text{ d}^{-1}$ ) in the second experiment was higher than that determined from Equation 1 ( $0.095 \text{ d}^{-1}$ ) indicating that the conventional method of calculating specific growth rates underestimates the specific growth rate as periods of non-exponential growth and lag phases are difficult to identify and exclude, respectively. During three days in the first experiment a specific growth rate of  $0.48 \text{ d}^{-1}$  was calculated from  $\ln(dx/dt)$ , which is

greater than previously recorded for *C. crispus* (Bird et al., 1979; Chopin et al., 1999; Neish et al. 1977; Simpson et al. 1977).

To compare specific growth rates from this study to those determined by others, we only compare specific growth rates found using Equation 1, which is the method commonly used. Hereby specific growth rates of *C. crispus* of  $0.066\text{ d}^{-1}$  and  $0.095\text{ d}^{-1}$  are determined for the first and second experiment, respectively. Simpson et al. (1977) found specific growth rates of *C. crispus* T4 strain of  $0.061\text{ d}^{-1}$  in tank cultures with supplement of ammonium, phosphate, and carbon dioxide. Neish et al. (1977) found specific growth rates reaching  $0.093\text{ d}^{-1}$  of the same strain in flow-through systems with supplement of nitrate and phosphate under continuous illumination in comparison to  $0.060\text{ d}^{-1}$  with solar illumination. The thalli of *C. crispus* used in our experiments are undefined and represent the natural population for the given area in Denmark, but the results are comparable with those obtained from the fast growing isolated T4 strain. However, the specific growth rates in our study were higher than those recorded elsewhere for natural *C. crispus* populations, which were between  $0.03\text{ d}^{-1}$  and  $0.04\text{ d}^{-1}$  (Chopin et al., 1999) and collected from a natural environment but tested in laboratory cultures in batch mode with weekly medium replacement reached specific growth rate of  $0.084\text{ d}^{-1}$  (Bird et al., 1979). The latter scientists found optimal temperature of  $15\text{ }^{\circ}\text{C}$ , light irradiance saturated above  $65\text{ }\mu\text{E m}^{-2}\text{ s}^{-1}$  and optimal salinity around 30 ‰ for *C. crispus*. The water temperature in our experiment was kept at  $20\text{ }^{\circ}\text{C}$ , which was the lowest possible with the heat from the light cabinet surrounding the photobioreactor, and the cooling capacity of the cooling tubes. This water temperature is what the macroalgae could be subjected to during summer in Denmark, and is in the range of  $14\text{--}20\text{ }^{\circ}\text{C}$  found by others for optimal growth of *C. crispus* (Bird et. al., 1979; Simpson and Shacklock, 1979; Juanes and McLachlan, 1992). The light intensity regulated by carbon dioxide addition and thereby metabolic activity was used for microalgae growth as increased metabolic activity increases self shading and thereby increases the demand for illumination (Eriksen et. al., 1996). This light regime tested on *C. crispus* in this investigation is different from previous studies with macroalgae. Usually, macroalgae are subjected to light and dark regimes, and during tank culture furthermore with intermittent light conditions caused by circulation of the culture with light at the water surface and darkness at the bottom of the tank (Pang and

Lüning, 2004; Lüning, 2005). In addition to findings by Bird et. al. (1979) of light intensity saturated above  $65 \mu\text{mol E m}^{-2} \text{s}^{-1}$ , outdoor tank studies show that *C. crispus* photosynthesis is saturated at  $100 \mu\text{mol E m}^{-2} \text{s}^{-1}$  (Cabello-Pasini et. al., 2000). This means that the thalli might have been light deficient at the beginning of our experiments with irradiance of  $20 \mu\text{mol E m}^{-2} \text{s}^{-1}$  and  $50 \mu\text{mol E m}^{-2} \text{s}^{-1}$  in the first and second experiment, respectively. The low irradiance in the beginning of the first experiment could have caused the relatively long lag phase before exponential growth initiated. However, the highest specific growth rate calculated from  $\ln(dx/dt)$  in the first experiment was initiated at an irradiance of  $30\text{--}40 \mu\text{mol E m}^{-2} \text{s}^{-1}$ , and the third and lower specific growth rate was during irradiance higher than  $200 \mu\text{mol E m}^{-2} \text{s}^{-1}$ .

This investigation with on-line data shows the importance of control of physiochemical variables, as the on-line data revealed a lag phase of 7 days before exponential growth was initiated in the first experiment, most likely because of low irradiance. Also the method identified non-exponential growth by the end of the second experiment most likely due to nutrient depletion. Tagged thalli of the second experiment had higher specific growth rates calculated from Equation 1 than those in the first experiment, but because of the physiochemical differences and few experiments we cannot conclude that fragments of thalli grow faster in the photobioreactor.

The tissue contained less dry matter in the second experiment than thalli in the first experiment, and this complies with the findings of Rivera-Carro et al. (1990), who showed that the stipe contained a higher percentage of dry weight than the apical tissue.

Phosphate was depleted earlier than nitrate by *C. crispus* in both experiments, but despite of this growth was most likely not P-limited, because inocula were stored the night before at high nutrient concentrations. A surge uptake of phosphate for the incorporation of polyphosphate is not likely according to Chopin et. al. (1997) as overcompensation is not recorded for *C. crispus*. According to the on-line data and phosphate measurements (Figure 5 and 7) the specific growth rate decreases 3 and 5 days after phosphate depletion in the first and second experiment, respectively. However this lower specific growth rate is probably not related to the phosphate depletion as studies show that P-limitation is not an important factor controlling photosynthetic metabolism (Chopin et. al., 1997).

Macro-epiphytes were located on the surface of older parts of thallus and stipe. This was also reported by Rosenvinge (1931) and Neish et al. (1977), but despite mechanical removal and chemical treatment, the *C. crispus* batch culture could not be kept clean of macroepiphytes in the first experiment. For comparison, a batch cultivation of *C. crispus* was investigated with thalli collected at the same location as in the first experiment on February 20<sup>th</sup> 2006, which showed massive growth of yellow microalgae contaminant (results not shown) despite the same chemical treatments as the first experiment. This most likely indicates that the chemical treatment of collected thalli needs to be optimized every time new thalli are collected as the contaminant flora varies with season. However, removal of the old stipe followed by the chemical treatment eliminated growth of macroepiphytes creating a batch culture without visible contamination of *C. crispus* in the second experiment.

Towards the end of the second experiment some morphological changes were observed. The algae were proliferating from the thallus surface as well as from the apical tissue and this vegetative regeneration most likely reflects the rapid growth and ability to generate biomass from unusual positions, under beneficial nutritional and physiochemical conditions.

In this study we have presented a novel macroalgal system, which provides on-line information about the metabolic activity. This method identifies different phases of growth unlike the commonly used method for specific growth rate calculations based on biomass increase throughout the entire experiment. The bubble column photobioreactor comprises a system in which specific growth rates of *C. crispus* are high. The closed system was validated by mass balancing accounting for all inputs and outputs. The harvest of freshly formed thalli was a simple method for generating macroalgae inoculum with a well defined history and free of macro-epiphytes. Further investigations are needed to exploit the potential of the closed system bubble photobioreactor with on-line registration and controlled variables for macroalgae, in order to understand the importance of each physiochemical variable and to increase productivity of biomass.

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