"GREEN CAVIAR" AND "SEA GRAPES": TARGETED CULTIVATION OF HIGH-VALUE SEAWEEDS FROM THE GENUS *CAULERPA*

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***Photo**: Sea grape production showing growth after 6 weeks with harvested section (front right)*

Executive Summary

This research project describes the first detailed and simultaneous examination of the aquaculture production and nutritional values of edible seaweeds in Australia. “Sea grapes” is a collective term for the edible varieties of the green seaweed genus *Caulerpa* that are harvested and consumed fresh in nations throughout the Pacific. These species are also present throughout Australia. However, only one species (*Caulerpa lentillifera*) is in aquaculture production in Japan and SE Asia, and it is unclear, to date, whether other sea grapes can also be domesticated or have comparable nutritional value.

Here we conduct comparative analyses of biomass productivity and nutritional composition of *C. lentillifera* (“green caviar”) and *C. racemosa* var. *laetevirens* from tropical Australia. We focused exclusively on these species for the empirical components as we found that other common varieties of sea grapes from the tropics (*C. racemosa* var. *racemosa*, Townsville) and sub-tropics (*C. geminate* & *C. sedoides*, Coffs Harbour) were not suited to aquaculture production via vegetative propagation. Commercial-scale production was evaluated using 1 m^2 (5 cm deep) culture units developed for vegetative propagation of *C. lentillifera.* This system operates at high stocking densities (>5 kg m^{-2}) and harvestable biomass protrudes through the top of the unit. Productivity of *C. lentillifera* in a 6 week cycle yielded, on average, 2 kg FW $week^{-1}$ and retained 6 kg m^{-2} stock within the unit. However, two consecutive 3 week culture cycles *C. racemos*a yielded <0.5 kg $week^{-1}$ of new growth above the unit, which did not compensate for loss of stock within the unit on both occasions (total biomass losses of up to 1.3 kg $week^{-1}$). Morphometric comparisons of the harvestable biomass revealed that *C. lentillifera* had a higher proportion of fronds to roots (68% *vs.* 48%), at a greater density per unit area (50 *vs.* 30 fronds cm^{-2}). *C. racemosa* fronds were significantly longer (6 cm *vs.* 3 cm), and therefore suited to a shorter culture cycle.

The nutritional value of the fronds (omega [ω]-3 & 6 fatty acids, antioxidant pigments and trace elements) was generally higher in *C. racemosa*. *C. racemosa* had higher unsaturated fatty acid contents (12 *vs.* 6 mg g^{-1} DW) and a slightly better ratio of ω-3: ω-6 (2 *vs.* 1.5). Trace elements varied substantially between the species (2 to 100-times), including higher levels in *C. lentillifera* of zinc (27.55 *vs.* 0.08 ppm), magnesium (16,650 *vs.* 4,115 ppm) and strontium (143 vs 0.16 ppm) and higher levels in *C. racemosa* of selenium (124.0 *vs.* 3.9 ppm). Some less desirable elements were higher in *C. lentillifera,* for example, arsenic (1 vs 0.1 ppm) and cadmium (0.53 *vs.* <0.05 ppm), whereas others were higher in *C. racemosa* including lead (4.45 *vs.* 0.16ppm), copper (7.19 *vs.* 0.89 ppm) and vanadium (10.14 *vs.* 0.44 ppm). *C. racemosa* had ~2 times the antioxidant content (chlorophyll a & b, β – carotene; 100 *vs.* 50 mg g^{-1} FW).

Overall *C. lentillifera* has high production rates and therefore warrants commercialisation as a new aquaculture product in Australia. On the other hand *C. racemosa* has many nutritional traits and some growth traits (e.g. frond length) that indicate potential for commercial production or alternatively for aquaculture ranching using wild harvests as a seedstock. The two species are both viable options for the establishment of a high-value, edible seaweed industry in Australia, which may be complimented by other sea grapes from the diverse genus of *Caulerpa* that can be found on all coastlines.

Introduction

Green seaweeds from the genus *Caulerpa*, particularly *C. lentillifera* and *C. racemosa* varieties, are consumed throughout the Pacific, where there is increasing pressure to address sustainability of harvest and rising market prices for domestic production (South 1993, Ostraff 2006). To date, commercial aquaculture production exists only for *C. lentillifera* (see Horstmann1983, Paul & de Nys 2008, Saito et al. 2010) which is also traded internationally (from the Philippines and Vietnam into Japan). However, the potential for aquaculture production of the numerous other varieties of *Caulerpa* sea grapes throughout the Pacific have rarely been evaluated (Paul & de Nys 2008), and never using high density, large-scale systems to enhance vegetative propagation of the biomass. The development of a practical commercial system for sea grape aquaculture will also enable control of the production cycle, both of biomass production and product quality. For example, productivity can be manipulated to enhance vertical growth of the shoots (or fronds) in high density cultivation, and, at the same time, influence the shape and texture of these fronds (Paul & de Nys 2008).

An opportunity also exists to link consistency in product quality with nutritional composition or value, as these traits frequently vary in wild harvested seaweeds (Galland-Irmouli et al. 1999). A consistent product quality would strengthen marketable health benefits, which is critical for whole food marketing and product value (e.g. Shahidi 2009). The key recognised nutritional components of seaweeds are protein, fatty acids, vitamins and other phytochemicals, and also minerals (Dawczynski et al. 2007a, MacArtain et al 2007, Bocanegra et al 2009, Holdt & Kraan 2011). With respect to crude protein, levels among different sea grape species in culture are similar (from 3.6 – 7.5% DW: Kjehdral conversion (N x 6.25): Paul & de Nys 2008), but are low compared to other seaweeds (19-44% DW:

Wong & Cheung 2000, Marsham et al 2007, Patarra et al 2011). However, the potential health benefits and nutraceutical properties of seaweeds extend beyond protein nutrition. For example, seaweeds and their extracts used in animal trials consistently mitigate serious health problems relating to atherosclerosis, heart and hepatic functions, presumably driven by antioxidants or fibre content (Huang et al. 2010). Similarly, seaweeds could be important sources of essential minerals or trace elements that may meet recommended daily intakes (Indegaard & Minsaas 1991, Ortega-Calvo et al. 1999, Rupérez 2002, Dawczynski et al. 2007b).

Polyunsaturated fatty acid (PUFA) and mineral contents are two functional and nutritional components that differentiate seaweeds from terrestrial food crops (Ortega-Calvo et al. 1999, Rupérez 2002, Bocanegra et al. 2009). Furthermore, an increasing number of studies using seaweeds have demonstrated health benefits from diet replacements or extracts (see Holdt & Kraan 2011), including the sea grape *C. lentillifera* (Matanjun et al. 2009). *C. lentillifera* has a relatively high content of polyunsaturated fatty acids (PUFA) at >5% of DW, including omega-3 (ω3) fatty acids such as linolenic acid 18:3 (Matanjun et al. 2009, Kumari et al. 2010, Saito et al. 2010). PUFAs and other phytochemicals presumably play important bioactive roles in antioxidant activity, even at low levels (Murata et al 1999, Bocanegra et al 2009). In addition, minerals are a major component of sea grape biomass. High mineral contents typically mean that important micronutrients (such as Zn and Fe) and essential trace elements (including Co, Cr, Mo, Ni, Se, and V) are available at levels that can meet daily requirements (Peña-Rodríguez et al. 2011). However, the concurrent bioaccumulation of other elements (including heavy metals Cd, Pb, and Sb or potentially problematic elements, eg. As, I) may balance or limit any perceived health benefits. Seaweeds naturally accumulate metals in their tissue, which can easily be compared to industry standards (Rupérez 2002), but should be quantified for quality assurance.

Because the majority of seaweed production is of red and brown seaweeds (Paul & Tseng 2012), direct comparisons of the nutritional value of green seaweeds from wild harvest and aquaculture produce are rare. There tends to be some consistency in fatty acid content between samples (e.g. for *Caulerpa lentillifera*: Saito et al. 2010) but often large differences in other aspects of nutrition (including mineral content: Peña-Rodríguez et al. 2011). Here we examine the links between aquaculture production and nutrition, simultaneously comparing the biomass productivity, fatty acid content, pigment content and mineral content of two species of sea grapes *C. lentillifera* and *C. racemosa* var. *laetevirens* in the controlled setting of high-density cultivation. As aquaculture production provides for options of continuous harvest, we also examine whether there is any influence of morphology and growth state that could inform production and harvest cycles to maximise nutritional benefits. The specific aims of this study were firstly to evaluate whether these sea grapes are amenable to high-density aquaculture production, and subsequently, to characterise the nutritional value under the same culture conditions. To do this we quantify the fatty aci content (targeting unsaturated fatty acids) and the main photosynthetic pigments (i.e. the antioxidant capacity) as well as characterising the mineral content of both beneficial and potentially problematic trace elements.

Materials and Methods

Biomass culture system

Caulerpa lentillifera and *Caulerpa racemosa* var. *laetevirens* were collected from Kissing Point, Townsville, and held in a circulating aquaculture system at the Marine and Aquaculture Research Facilities Unit, James Cook University (JCU), Townsville, Australia. The system was integrated with abalone and sea urchins (marine herbivores) providing nutrient levels on average 1 mg L^{-1} nitrogen in the 25,000L capacity system.

The culture vessels used were open raceways (1m * 2m * 0.2m: W*L*H) which generate unidirectional flow using a tip bucket (8L) at the inlet to provide pulsed and turbulent motion (~60 s frequency). Water exchange was maintained at ~1 volume (400L) per hour. Prior to the experimental period, optimum stocking densities and harvest culture cycles of *C. lentillifera* and *C. racemosa* were evaluated to select a preferred cycle for each rather than standardising growth cycles between species. *C. lentillifera* was trialled with initial densities of 4 – 6 kg m^{-2} from 0 – 6 weeks over three months (20 culture trials). The selected stocking density and growth cycle was 6 kg m^{-2} and 6 weeks. We found that *C. racemosa* was not suited to high stocking density (i.e. >4 kg m^{-2}) nor long culture periods (>3 weeks) and was instead trialled between 2 – 4 kg m^{-2} (8 culture trials). The selected stocking density and growth cycle was 3 kg m^{-2} and 3 weeks.

Environmental variables were recorded throughout the experimental period. Diurnal changes in surface PAR were recorded at three times (weeks 1, 7 and 16) and the maximal (1200 hr) surface PAR was measured weekly. Surface PAR peaked at 1200 and averaged 170± 50 µmol photons m^{-2} s^{-1} (mean ±1SD) for the duration of the experiment. All forms of

nitrogen (ammonia, nitrite, and nitrate) were quantified at the beginning of the experiment but only nitrate was monitored thereafter, using a Hach Colorimeter. During the experimental period nitrogen (NO_3^- - N) was, on average, 1.8 $\pm$ 0.4 mg L^{-1}, temperature was 27.2 $\pm$ 1.3 °C, salinity was 36.7 $\pm$ 0.6 ppt and pH was 8.14 $\pm$ 0.04.

Biomass Production

Production yields of *C. lentillifera* and *C. racemosa* were evaluated both in monoculture and in co-culture. Co-cultures were evaluated as both species appeared to grow well when stocked in the same tray and this co-culture concept had not previously been evaluated. Biomass was enclosed within a culture vessel following methods developed and patented by James Cook University (Paul & de Nys 2011; see also Fig. 1a-c). The vessels were square (0.9*0.9m, 6cm deep) perforated plastic trays (halved RV6 Aquatrays, Tooltech Pty Ltd, Brisbane). Culture trays were rotated every 3.5 days, moving randomly amongst positions with the system. Algae were weighed weekly (to $\pm$ 0.1 kg) by suspending culture trays from a spring balance to a steady weight.

Each species was treated differently based on the previous growth trials (see above). One trial was run for *C. lentillifera* for 6 weeks, whereas two separate 3 week cycles within the same 6 week period was used for *C. racemosa* and the co-culture. The initial stocking density for *C. lentillifera* was 6 kg m^{-2}, for the co-culture was 3 kg of each, and for *C. racemosa* was 3 kg m^{-2} for the 1st culture period. For the second culture period, the initial stocking density of *C. racemosa* was decreased by half to 1.5 kg for both the monoculture and co-culture (1.5 kg + 4.5 kg for *C. racemosa* and *C. lentillifera*, respectively). Biomass production yields were plotted overtime.

Biomass properties

Morphometrics were analysed by comparing the percentage of harvestable biomass that were fronds, as well as the frond density (# fronds per 100cm^2) and frond lengths (cm) both between species and within species between monoculture and co-culture. The final proportion of harvestable biomass (above tray biomass) was measured at the end of each experimental period by haphazardly sampling (n = 3 individual clumps per tray) typically of 40-80 g fresh weight. Frond density and frond length were quantified mid-period after 2 weeks when the biomass had become established. Frond density was measured as fronds protruding above the tray per 100 cm^2 using the average of the 4 quadrants of each tray (with n = 4 sub-samples of 25 cm^2 in each quadrant). Average frond lengths were measured at the same time for 3 randomly selected fronds within each quadrant (n = 12 fronds per tray).

Sub-samples of fronds from *C. lentillifera* and *C. racemosa* were subsequently harvested for analyses of the nutritional components using samples from the monocultures of each species. For fatty acid and pigment analyses, fronds of different lengths of both *C. lentillifera* and *C. racemosa* (2 cm – 10 cm) were selected haphazardly from the biomass, spun-dry and weighed. Individual fronds were then snap frozen in liquid nitrogen and freeze-dried under dark conditions. All freeze-dried biomass was stored at -80°C until extraction. For elemental analyses, multiple fronds of different sizes were combined and oven dried at 60°C for 2 days. The calorific value of each species (n = 2 samples) was quantified using a BOM calorimeter.

Nutritional Properties - Fatty acid analysis

A direct transesterification method adapted from Carvalho and Malcata (2005) and Cohen et al. (1988) was used to simultaneously extract and esterify the fatty acids (as methyl esters). 30 mg samples of freeze-dried biomass of both *C. lentillifera* and *C. racemosa* were extracted with 2 ml methylation mixture (methanol:acetyl chloride, 20:1 *v*/*v*) and 300 µl internal standard solution (nonadecanoic acid, 0.2 mg ml^{-1} in methanol). The samples were heated at 100°C for 60 minutes, cooled to room temperature to add 1 ml extraction solvent (Hexane with 0.01% BHT w/w), and then heated again to form a single phase. Samples were again cooled and 1 ml of milli-Q purified water added to facilitate phase separation. The hexane (upper) phase was collected and filtered through a 0.2 µm PTFE syringe filter prior to analysis.

Fatty acid analysis was carried out using gas chromatography – mass spectrometry (GC-MS) in scan-mode on an Agilent 7890 GC equipped with a flame ionization detector (FID) and connected to an Agilent 5975C Electron Ionisation (EI) Turbo Mass Spectrometer (Agilent Technologies Australia Pty Ltd). Separation was achieved on a DB-23 capillary column (cyanopropyl stationary phase, 60m x 0.25 mm id x 0.15 µm) with helium as the carrier gas. Injector and FID inlet temperatures were 150 °C and 250 °C, respectively (split injection, 1/50). Column temperature was held at 50 °C for 1 min, then raised linearly at 25 °C min^{-1} to 175 °C, followed by a 4 °C min^{-1} increase to 235 °C, and a 3 °C min^{-1} increase to 250 °C (following David et al. 2002). The quantity of fatty acids was determined by comparison of peak areas of authentic external standards (Sigma Aldrich), and was corrected for recovery of internal standard (C19:0). Total fatty acid content was determined as the sum of all fatty acid methyl esters. Fronds of *C. lentillifera* and *C. racemosa* ranging from 1-9 cm were analysed (n = 9 & 11, respectively).

Nutritional Properties - Pigment analysis

Freeze-dried samples were incubated in chilled extraction solvent (99% methanol [MeOH], 1% 0.5 M tetrabutylammonium acetate [TBAA]) in the dark for 2h, followed by filtration (0.2 µm, Econofilter PTFE membrane, Pacific Labs) as modified from van Heukelem and Thomas (2001). Pigment extracts were analysed on a Varian Prostar HPLC, combined with a Varian Prostar UV-Viz detector (monitoring at 440 nm) and a 3.5 µm, 4.6 x 150 mm C-8 Agilent Eclipse XDB column (Agilent, Australia). A two solvent gradient with a flow rate of 1.1 ml min^{-1} was used to separate the pigments at 60°C. Solvent A: 70:30 (v/v) MeOH:28 mM aqueous TBAA, adjusted to pH 6.5, and solvent B: 100% MeOH. The proportion of solvent B was 5% at t = 0 min, rising linearly to 50% at 15 min and held at 50% until 20 min when it was linearly increased to 100% at 38 min, then linearly returned to 5% at 40 min and maintained at 5% until 45 min. The peaks reported were identified by comparison of retention times and co-elution with authentic pigment standards obtained from the International Agency for ^{14}C Determination (DHI, Denmark). Pigments were quantified using response factors calculated from calibration curves of external standards. Fronds of *C. lentillifera* and *C. racemosa* ranging from 1-12 cm were analysed (n = 16 & 12, respectively).

Nutritional Properties - Mineral analysis

Elemental composition was taken at the end of the 6 week experiment, using biomass from monocultures of *C. lentillifera* and remaining biomass from *C. racemosa* (combining biomass from monocultures and co-culture). The concentrations of 21 different elements, listed in Table 5, were determined for the algae grown in the two treatments. 100 mg samples of the dried seaweed were placed into digestion vessels with 2.5 ml SupraPure (Merck Germany) double distilled HNO_3 and 1.0 ml AR Grade H_2O_2. The mixture was left to stand in the fume-

hood for two hours to allow the reaction to complete. The vessels were then heated to 180°C in a microwave oven (Milestone Starter D) and maintained at this temperature for ten minutes. After cooling to room temperature, the digested samples were diluted to 100 ml with Milli-Q water in a volumetric flask. Sample analysis was carried out using two instruments. Major elements (Al, Ca, K, Na and P) were measured using a Varian Liberty Series II Inductively Coupled Plasma Optical Emission Spectrometer (Melbourne, Australia). The remaining elements were measured using a Varian 820-MS Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (Melbourne, Australia). External calibration strategy was used for both instruments with a series of multi-element standard solution containing all the elements of interest and the results were reported after subtracting the procedure blanks. These analyses were done by the Advanced Analytical Centre at JCU. Two samples of *C. lentillifera* and *C. racemosa* were analysed.

Statistical analyses

Because of the variation in culture cycles and optimal initial stocking densities, the production experiments could not be formally analysed using ANOVA. Formal comparisons of the morphometric differences between species were made for monocultures, and then separately for each species comparing the monoculture to co-culture (as co-culture data for individual trays are not independent). We used separate 3-factor nested ANOVAs to compare frond lengths between "Species" in monoculture and within each species for "Culture type" (mono- *vs.* co-culture), also comparing in each analysis the hierarchical sub-sampling of Tank(Species or Culture type) and Quadrat(Tank) (see statistical outputs in Table 6 for details).

All fatty acid and pigment content data (mg g^{-1}) were analysed using ANCOVA with species as the fixed factor and frond length (cm) as the covariate. The ANCOVA assumptions of homogeneity of variance and normality were examined using scatterplots of residuals versus predicted values and histograms of residuals, respectively. The additional assumption of co-linearity for ANCOVA was examined by running the full model with Species x Frond Length. Frond Length for both *C. lentillifera* and *C. racemosa* were consistently in the same range 1 – 12 cm. Specific fatty acids (α-linolenic acid [ALA] and eicosapentenoic acid [EPA]) and pigments (chlorophyll-b and β-carotene), as well as summary data of fatty acids (total fatty acids, total ω-3), were analysed in separate ANCOVAs.

Results & Discussion

Biomass Production

Total biomass productivities of the monocultures were very high for *C. lentillifera*. After 1 week of acclimation to the system, growth was consistent with the following 5 weeks producing on average 2 kg week^{-1} (Fig. 2). This translated to an average "above tray" mass of 1.5 kg week^{-1} (i.e. new or harvestable biomass) and after 6 weeks the harvestable portion equated to 65% of the total biomass (Table 2; total biomass increased, on average, to 16.9 kg ± 0.4 SE). The culture system used a tip bucket to generate sporadic and turbulent water renewals and this was a key feature to sustaining biomass productivity with high biomass densities of up to 12 kg m^{-2} for this benthic seaweed, most likely by breaking boundary layers and facilitating nutrient transfer from the water column (Hurd 2000). In contrast, *C. racemosa* appears to be less amenable to high density culture, as the highest total biomass productivities for monocultures of *C. racemosa* over a 3 week period were net negative at minus 0.45 kg week^{-1} (Table 1). The above tray biomass averaged up to 0.28 kg week^{-1} (1st period, 0–3 weeks); however, total biomass decreased on average from 3 kg to 0.8 kg ± 0.11 SE (1st period, 0–3 weeks) and 1.5 kg to 0.5 kg (2nd period, 4–6 weeks).

Co-cultures provided an alternative option for the production of *C. racemosa.* The biomass productivity of *C. racemosa* co-cultures was similar to monocultures with the same initial stocking density in both experimental periods (Fig. 2). Competition did not alter the biomass production of *C. racemosa* above the tray in the first experimental period (Fig. 2), growing at 0.28 kg week^{-1} ± 0.13 SE (monoculture) and at 0.23 kg week^{-1} ± 0.06 SE (co-culture). However, at a lower stocking density in the second experiment period, biomass production decreased more in monoculture (-0.027kg week^{-1} ± 0.01 SE) than in co-culture (-0.06 kg

week^{-1} ± 0.01 SE). Co-cultures also influenced the biomass productivities of *C. lentillifera,* as competition with *C. racemosa* appeared to delay establishment. This lead to a reduction in growth of *C. lentillifera* in the first 3 weeks of both experimental periods, regardless of stocking density (Fig. 2). These results highlight that different species of sea grapes will require different stocking densities for commercial production, and that these are also influenced by potential competitive interactions with other seaweeds if they are not maintained in monoculture.

Biomass Properties

The above tray biomass production is the harvestable portion and is the best metric for biomass quality (see Fig. 1c, and cover image). The proportion of edible fronds was typically higher in *C. lentillifera* than *C. racemosa* (Fig. 3). Co-culture of *C. racemosa* achieved larger portions of edible fronds than in monoculture in both the first (71.5% *vs.* 57.6%) and second experimental period (54.8% *vs.* 47.8%). The decrease in overall proportion between the two experimental periods correlated with the decreased initial stocking density of 3 kg (Exp. 1) *vs.* 1.5 kg (Exp. 2). The quality of the biomass was also altered by the type of culture, as the density of the fronds was lower in co-cultures for both species (Fig. 4a). However, although the frond density of *C. lentillifera* in monoculture was typically higher than in co-culture and than *C. racemosa*, it also had the highest variance of any treatment (Fig. 4a).

The fronds of monocultures of *C. lentillifera* were half the length of fronds from monocultures of *C. racemosa* (Fig. 4b: Table 6, ANOVA, *Species*, p = 0.006). The vertical growth of fronds of *C. racemosa* was staggering at, on average, 0.42 cm per day (0.02 SD) and > 1 cm per day in some instances. The largest frond recorded during random sampling at the 2 week sample point was 20.8 cm in the *C. racemosa* monoculture. *C. lentillifera* was on

the other hand slower in vertical growth at 0.21 cm per day (0.01 SD) but up to 0.5 cm per day. However, individual fronds of *C. lentillifera* were almost double the mass of *C. racemosa* (0.54 g *vs.* 0.27 g after 3 weeks). There were no effect of co-culture on the biomass properties of *C. racemosa* (Table 5, ANOVA, 'Culture type', p = 0.361) but there was an effect on the fronds of *C. lentillifera* (Table 5, ANOVA, 'Culture type', p = 0.048). *C. lentillifera* in co-culture increased in frond length by 25% when, at the same time, biomass production of *C. lentillifera* was lower than monoculture (Fig. 4b & Fig. 2 above). These results indicate that there is considerable variation between fronds within culture types and between species, and highlights that frond length is a variable that could be manipulated in culture to meet desired specifications.

The wet:dry weight ratios of the fronds was the same, on average, for both species *C. lentillifera* at 21.3:1 (1.3 SD) compared to 21.3:1 (1.2 SD) for *C. racemosa*. There was some variability between replicate samples for each species (18.3 – 25.1 for *C. lentillifera*; 20.0 – 23.4 for *C. racemosa*), although this variation was not correlated to the size of the fronds. Given that sea grapes are siphonous in structure, i.e. essentially one continuous multi-nucleate cell, it is possible that the differences in wet:dry weight ratios between fronds is a result of within-plant partitioning of resources. This in turn suggests that there may also be within-plant variation in the nutritional quality of fronds. Variation in nutritional quality at this scale has not previously been reported for seaweeds, but could identify sources of variation in product quality or particular traits that can be targeted during cultivation.

Nutritional Properties - Fatty Acid Analysis

The total fatty acid content was relatively low in both species of sea grapes below 30 mg g^{-1} (Table 3). This compares with other species of green seaweeds from the same taxonomic

grouping of siphonous algae (Bryopsidales) of 40 mg g^{-1} for *Caulerpa taxifolia* and 70 mg g^{-1} for *Derbesia tenuissima* (Gosch et al. 2012). However, the relatively high levels of polyunsaturated fatty acids in these species (>50% of the total fatty acid content) compared to other studies on wild-harvest sea grapes (e.g. Kumari et al. 2010 with 27.2%) indicates that aquaculture product can differ to wild harvests (see also 16:3 is high in *C. lentillifera*; Saito et al. 2010). There have been a significant number of studies that have screened fatty acid content and mineral contents of wild harvest seaweeds (Matanjun et al. 2009, Kumari et al. 2010). However, the environmental differences between species cannot be partitioned unless the different sea grapes are compared under same conditions. We show here that there are clear differences between species in controlled conditions (there is almost double the total fatty acid and PUFA concentrations in *C. racemosa*; Fig. 6a) but importantly also demonstrate that there is large variation in the concentrations of specific fatty acids within an individual (Fig. 6b-c).

Within-plant variation in sea grapes is best demonstrated by the two major omega-3 fatty acids α-linolenic acid (ALA) and eicosapentenoic acid (EPA). These fatty acids are important for health and nutrition, and are key considerations for nutraceutical applications of algae (Bocanegra et al. 2010). We show that ALA was not influenced by frond size (Fig. 6b: ANCOVA, frond size, $F_{1,17} = 0.025$, $p = 0.876$); however, EPA significantly decreased with frond size and this effect was most pronounced for *C. racemosa* (Fig. 6c: ANCOVA, frond size, $F_{1,17} = 23.51$, $p < 0.001$). This means that frond height could be manipulated in culture to ensure that target fatty acids (such as EPA) are enhanced in the biomass by maintaining shorter culture cycles with correspondingly smaller fronds at harvest. Furthermore, the fatty acid nutritional value of *C. racemosa* appears to be better on all accounts than *C. lentillifera*. The mean concentration of ALA in *C. lentillifera* was 1.72 mg g^{-1} (±0.18 SE) half that of *C. racemosa* (4.24 mg g^{-1} ±0.19 SE). Similarly, the concentration in *C. lentillifera* was almost a

third of *C. racemosa* (0.18 ±0.01 SE *vs.* 0.52 ±0.05 SE). A ratio of omega-3/6 fatty acids of two is also an important dietary consideration due to the prevalence of omega-6 in western diets (Simopoulos 2002). *C. racemosa* had an omega-3/6 of 2 compared to that of *C. lentillifera* at 1.5 (Table 3). However, both species have relatively good fatty acid profiles (i.e. %PUFA of >50% of total fatty acids) and also high concentrations of PUFA compared to other sea grapes (Kumar et al. 2011).

Nutritional Properties - Pigment Analysis

Within-plant variation in the specific pigments varied in magnitude between and within species, similar to the omega-3 fatty acids (above). The total pigment content of *C. racemosa* (9.4 mg g^{-1}) was more than twice that of *C. lentillifera* (Fig. 6a, Table 5), and each of the three pigments analysed (chlorophyll-a, chlorophyll-b and β-carotene) were significantly higher in *C. racemosa* (Table 8, $p < 0.001$ for all). Variation in the specific pigments within species is best demonstrated by the chlorophyll-b and β-carotene (Fig 6b & c). These pigments differ in structure but are both important nutritionally as they have antioxidant and anti-cancer properties (Ortega-Calvo et al. 1999 Ferruzzi et al. 2002). We have shown that chlorophyll – b content was not influenced by frond length (Fig. 6b: ANCOVA, frond size, $F_{1,27}= 0.159$, $p = 0.386$) whereas β-carotene significantly decreased with frond length (Fig. 6c: ANCOVA, frond length, $F_{1,25} = 9.31$, $p = 0.005$). This was most pronounced for *C. racemosa*, for which longer fronds of 10cm had half the content of fronds <2 cm (Fig. 6c).

Chlorophyll is by far the most abundant pigments in sea grapes, more than 20-times the content of β-carotene (Table 4). Chlorophyll is an established antioxidant (Ortega-Calvo et al. 1999) with demonstrated anti-cancer properties (Ferruzzi et al. 2002). Furthermore, chlorophyll is used as a natural colour additive in food and pharmaceuticals (Rangel-Yagui et

al. 2004). However, much of the interest in antioxidants from algae has focussed on the carotenoids, primarily from microalgae such as diatoms (Garcia-Gonzalez et al. 2005). This study and others have found significant variation in β-carotene over short temporal scales, i.e. hours, and this variation appears to be positively correlated with cell growth (Garcia-Gonzalez et al. 2005). Here we show the opposite for sea grapes; that longer fronds tend to have lower contents of β-carotene (Fig. 6c). Furthermore, although the β-carotene content in our study was low compared to chlorophyll (Table 4) and much lower than reported values in microalgae (of up to 100 mg g^{-1}: Garcia-Gonzalez et al. 2005), it was still twice as much compared to the only other study on sea grapes from wild harvest biomass (Mantanjun et al. 2010). Therefore this variation in pigments related to culture conditions and morphometrics indicate that, similar to the fatty acids above, aquaculture production of sea grapes could focus on generating smaller fronds to maximise the potential health benefits. Taken together, the pigment and fatty acid nutritional properties are compelling and, when combined with essential trace elements, confirm that sea grapes are a whole food with a suite of functional components.

Nutritional Properties - Mineral analysis

The high mineral content of seaweeds has both positive and negative implications for nutrition. We have shown that both varieties of sea grapes have very high mineral contents, up to 23% of the dry weight (Table 5). This value is close to maximum levels for food crops, for example, algal products in the United States must be under 45% ash (total mineral content) and below 40 ppm heavy metals (USA Food and Nutrition Board, 1981). Under this scenario the heavy metal content *C. racemosa* would be acceptable whereas *C. lentillifera* had higher than acceptable levels (163 ppm, Table 5). There was also, however, substantial variation in the specific elements between species. For this reason such general conclusions

based on ash and summed metals may not be appropriate for sea grapes. Our elemental results indicate that the sea grapes had different affinities for elements, both potentially problematic and beneficial (Table 2), which means that they cannot be easily characterised as either positive or negative as it is difficult to attribute the relative importance of individual elements in human diets. Given that sea grapes are staple foods that have stood the test of time in many Pacific nations (South 1993, Ostraff 2006, Saito et al. 2010), it seems likely that these elements are also below problematic levels for human consumption.

None of the main essential minerals in sea grapes consumed in 100 g fresh portions (see Table 5) would meet the daily intake for adults of trace elements such as iron (10–18 mg), zinc (15 mg), manganese (2.5–5 mg) and copper (2–3 mg) (Indegaard & Minsaas, 1991). However, zinc was particularly high in *C. lentillifera* (27.55 *vs*. 0.08 ppm) and therefore could supplement other dietary intakes. While it is convenient to think that sea grapes, or seaweeds more generally, could satisfy dietary intake of a diverse range of minerals because of their ocean heritage, it is important to understand that the portions of edible seaweeds are often small and that even more established edible brown seaweeds, such as kelps, are similar in composition to sea grapes (see McArtain et al. 2007, Mantanjun et al. 2009). Brown seaweeds may in fact only contribute iodine as a unique mineral feature (Dawczynski et al. 2007b), yet ironically iodine also represents one of the main concerns with seaweed consumption in the general public. However, this concern should be limited to brown seaweeds, including kelps and *Sargassum*, as *Caulerpa* has relatively low levels of iodine (Matanjun et al. 2009). Similarly, other known carcinogens such as arsenic can be high in specific brown seaweeds (18-124 ppm: Rose et al. 2007) but was relatively low in our study at 1 ppm (Table 5).

Therefore sea grapes can certainly be considered as a nutritional food but not as a functional food based on its nutritional components. Independently, the PUFA content, chlorophyll content and the essential trace elements in sea grapes are not unique from other seaweeds or plants. The ability to make claims linking the biochemical composition of sea grapes with functional properties of whole foods requires a different series of evaluations against animal models or similar (Shahidi 2009, Holdt & Kraan 2011). However, sea grapes also have a pleasant sea flavour, an ornate structure with brilliant emerald colour, as well as novelty texture from bursting "lentil"-like branchlets when consumed (see Fig. 1b), and these are perhaps more compelling features upon which to focus than any added benefits to nutrition.

Conclusions

Caulerpa is diverse seaweed genus that is common in tropical and temperate environments throughout Australia. It also has diverse morphologies and the sea grape varieties have large potential to be more widely consumed as a sea salad. We have demonstrated that the most important traits for aquaculture production of sea grapes are the ability to grow rapidly from vegetative fragments which are stocked at high stocking densities in land-based facilities. The culture system must importantly be controlled to deliver water motion that facilitates the above-tray growth of the biomass for harvest. These features are critical for the successful commercial production of sea grapes. *C. lentillifera* represents the most suitable sea grape for development of a fresh, edible seaweed industry in Australia. Not all species of *Caulerpa* are suitable for consumption, and it is notable that *C. lentillifera* and other sea grapes have lower concentrations of secondary metabolites than the feather-like species (e.g. *C. taxifolia* and *C. sertularioides*: Baumgartner et al. 2009). Correspondingly the sea grape varieties are not bitter in taste but have a more subtle sea flavour. However, not all species of sea grapes are amenable to aquaculture cultivation.

Mass cultivation of seaweeds faces numerous challenges in scalability of productivity and quality (Lüning & Pang 2003). However, aquaculture also provides the opportunity to create a uniform product under controlled conditions, with the added benefit of sustainable production by reducing the reliance on wild harvests. We also demonstrate that aquaculture can be used to manage the production cycles to consistently produce and harvest fronds of shorter length that maximise the nutritional profiles. Links between variation in morphology and biochemical composition have, until now, been overlooked – yet the ability to manipulate these traits could enable any future industry to diversify products and enhance marketability of the product for health and lifestyle. The vast majority of global seaweed production is focussed on dried products from large-scale oceanic culture in China and Korea (Lüning & Pang 2003, Paul & Tseng 2012). If fresh seaweed production can instead be decentralised and located closer to market, then commercialisation in regional areas of Australia could be achieved for these products. Integrating with existing land-based aquaculture facilities offers the opportunity to cost-share by using nutrient waste streams and associated infrastructure.

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Tables 1 – 8

Table 1. Summary of biomass production and properties of *Caulerpa lentillifera* and *C. racemosa*. Data show mean biomass productivities and biomass properties (± 1 SE).

Attribute	*Caulerpa lentillifera*	*Caulerpa racemosa*
Biomass Production		
Total Biomass	2 kg week^{-1} (6 week cycle)	-0.45kg week^{-1} (3 week cycle)
Above Tray Biomass	1.5 kg week	0.1kg week
Harvestable proportion	65%	10%
Biomass properties		
Proportion of Biomass	68%	48%
Frond Density	50 fronds per 100 cm^2	30 fronds per 100 cm^2
Frond Length	3 cm (per 2 weeks)	6 cm (per 2 weeks)
FW:DW	21.3	20.9

Table 2. Summary of nutritional properties of *Caulerpa lentillifera* and *C. racemosa***.** Data show mean nutritional properties (± 1 SE). Indicated in **green** are potentially beneficial levels of positively perceived minerals and **red** are potentially problematic levels of negatively perceived minerals.

Nutritional Property	*Caulerpa lentillifera*	*Caulerpa racemosa*
Lipid		
Total FAs	11.2 mg g^{-1}	22.5 mg g^{-1}
ω-3	3.2 mg g^{-1}	7.0 mg g^{-1}
ω-6	2.1 mg g^{-1}	3.6 mg g^{-1}
ω-3:ω-6	1.5	2.0
Saturated	40.8%	43.3%
Mono-unsaturated	12.0%	9.7%
Poly-unsaturated	47.4%	47.0%
Variance with frond size	None to Negative	None to Strong Negative
Pigment (antioxidant)		
Chlorophyll – a	2.58 mg g^{-1}	5.48 mg g^{-1}
Chlorophyll – b	1.45 mg g^{-1}	3.06 mg g^{-1}
β – Carotene	0.15 mg g^{-1}	0.39 mg g^{-1}
Variance with frond size	Strong negative	Weak negative
Energy	3.42 MJ kg^{-1} (0.16 MJ kg^{-1} FW)	7.08 MJ kg^{-1} (0.34 MJ kg^{-1} FW)
Elemental Composition		
Positive	ppm	ppm
Boron	18.4	14.4

Calcium	5,875	5,640
Magnesium	**16,650**	4,115
Selenium	3.90	**123.95**
Strontium	**143.00**	0.16
Zinc	**27.55**	0.08
Negative (acceptable ppm)	ppm	ppm
Arsenic (1)	1.06	1.17
Lead (0.3-0.8)	0.16	**4.45**
Cadmium (0.1)	**0.53**	<0.05
Copper (2-3 mg day^{-1})	0.89	7.19
Vanadium	0.44	**10.14**

Table 3. Fatty acid composition of *Caulerpa lentillifera* and *C. racemosa*. Data show mean concentration (mg g^{-1} of dry material ± 1 SE).

FAME concentration (mg g^{-1})		
FAME	*C. lentillifera*	*C. racemosa*
C14:0	0.35 ± 0.03	0.59 ± 0.03
C16:0	4.22 ± 0.36	9.15 ± 0.31
C16:1	0.84 ± 0.10	0.99 ± 0.07
C16:2 (n-6)	0.62 ± 0.04	0.78 ± 0.04
C16:3 (n-3)	1.35 ± 0.14	2.27 ± 0.10
C18:1t (n-9)	0.28 ± 0.02	0.54 ± 0.05
C18:1c (n-9)	0.22 ± 0.01	0.64 ± 0.06
C18:2 (n-6)	1.33 ± 0.10	2.30 ± 0.13
C18:3 (n-6)	0.18 ± 0.01	0.48 ± 0.04
C18:3 (n-3)	1.65 ± 0.17	4.24 ± 0.19
C20:5 (n-3)	0.18 ± 0.01	0.52 ± 0.05
FAME properties		
Total FAs (mg g^{-1})	11.2	22.5
SFA [wt%]	40.7	43.3
MUFA [wt%]	12.0	9.7
PUFA [wt%]	47.3	47.0
PUFA ω6 (mg g^{-1})	2.1	3.6
PUFA ω3 (mg g^{-1})	3.2	7.0
ω6:ω3	1.5:1	2.0:1

Table 4. Pigment composition of *Caulerpa lentillifera* and *C. racemosa*. Data show mean concentration (mg g^{-1} of dry material ± 1 SE).

Pigment concentration (mg g^{-1})		
Pigment	*C. lentillifera*	*C. racemosa*
Chlorophyll – a	2.58 ± 0.25	5.77 ± 0.45
Chlorophyll – b	1.47 ± 0.14	3.22 ± 0.19
β – Carotene	0.15 ± 0.01	0.42 ± 0.03
Pigment properties		
Total Pigments	4.2	9.4
Total Chlorophyll	4.1	9.0
Chlorophyll:Carotene	27.5	21.5

Table 5. Elemental composition of *Caulerpa lentillifera* and *C. racemosa*. Data show mean concentration (mg kg^{-1} [=ppm] of dry material dry material ± 1 SE). Note some elements were not detectable (**<**). Total HM/M (heavy metal/metalloid) content is the sum of Al, As, Cd, Cr, Pb, Sr, V. Conversion to fresh weight content (FW:DW) can be made using *C. lentillifera* (20:1) and *C. racemosa* (21:1). A typical portion of sea grapes for consumption is 100 g FW, equivalent to ~5g DW.

Elemental Composition (mg kg^{-1})				
Element	*C. lentillifera*		*C. racemosa*	
Aluminium	16.45	±1.15	7.19	±4.01
Arsenic	1.06	±0.11	1.17	±0.05
Boron	18.40	±0.90	14.40	±1.70
Calcium	5,875.00	±55.00	5,640.00	±40.00
Cadmium	0.53	±0.03	<0.05	
Chromium	1.60	±0.04	1.15	±0.04
Copper	0.89	±0.40	7.19	
Lead	0.16	±0.02	4.45	
Magnesium	16,650.00	±250.00	4,115.00	±615.00
Manganese	3.21	±1.39	3.83	±0.36
Mercury	<5.00		<5.00	
Molybdenum	<0.10		<0.10	
Nickel	<0.10		<0.10	
Phosphorus	<1000.00		851.0	
Potassium	7,410.00		<500.00	
Sodium	160,500.00	±1500.00	219,000.00	±4000.00
Selenium	3.90	±0.83	123.95	±0.26
Strontium	143.00		0.16	±25.05
Vanadium	0.44	±0.11	10.14	±0.05
Zinc	27.55	±6.45	0.08	±4.01
Elemental properties				
Total HM/M	163.24		24.26	
Total content (% dw)	19.1%		23.0%	
Na: K	21.7		>100	

Table 6. ANOVA results for comparisons of frond lengths of *Caulerpa lentillifera* and *C. racemosa* between monoculture, and between monoculture and co-culture for each species. Data were ln-transformed.

Source	**df**	**MS**	**F**	**p**
Monocultures				
Species	1	8.73	27.10	0.006
Tank (Species)	4	0.32	2.33	0.066
Quadrat (Tank)	9	0.14	0.99	0.458
Error	57	0.14		
C. lentillifera				
Culture type	1	0.83	10.45	0.048
Tank (Culture type)	3	0.08	0.33	0.803
Quadrat (Tank)	9	0.24	1.94	0.070
Error	46	0.12		
C. racemosa				
Culture type	1	0.459	1.15	0.361
Tank (Culture type)	3	0.398	7.65	0.008
Quadrat (Tank)	9	0.052	0.36	0.949
Error	45	0.145		

Table 7. ANCOVA results for comparisons of fatty acid contents of *Caulerpa lentillifera* and *C. racemosa* related to frond length. All data were ln-transformed.

Source	df	MS	F	p
Total FA				
Species	1	2.308	48.34	<0.001
Frond Length	1	0.001	0.03	0.872
Error	17	0.048		
α-Linolenic acid (18:3)				
Species	1	4.350	50.19	<0.001
Frond Length	1	0.002	0.03	0.876
Error	17	0.087		
Eicosapentanoic acid (20:5)				
Species	1	5.975	180.14	<0.001
Frond Length	1	0.747	23.52	<0.001
Error	17	0.032		

Table 8. ANCOVA results for comparisons of pigment contents of *Caulerpa lentillifera* and *C. racemosa* related to frond length. Chlorophyll-a data were ln-transformed.

Source	df	MS	F	p
Chlorophyll-a				
Species	1	5.151	21.79	<0.001
Frond Length	1	0.008	0.04	0.853
Error	25	0.236		
Chlorophyll-b				
Species	1	4.885	23.84	<0.001
Frond Length	1	0.159	0.78	0.386
Error	27	0.205		
β-carotene				
Species	1	7.408	77.67	<0.001
Frond Length	1	0.888	9.31	0.005
Error	25	0.095		

Figures legends 1 – 6

Figure 1. Habit of *Caulerpa* "sea grapes" in cultivation. a) Initial growth (1 week) of fronds emerging through the top of the tray, *Caulerpa lentillifera* monoculture. b) Close up of fronds of *C. lentillifera* showing the horizontal runners (stolons) wrapping over the top of the aquaculture tray and young fronds with "lentil"-like branchlets on opposite sides of the axis (white arrows). c) Close up of fronds of *C. lentillifera* and *C. racemosa* (white arrows) in co-culture.

Figure 2. a) Change in biomass (mean ± SE) of *Caulerpa lentillifera* and *C. racemosa* in monoculture and co-culture over time (weeks). Cultures initially stocked with 6 kg (*C. lentillifera* monoculture 0-6 wks), 3 kg *C. racemosa* "monoculture 0-3 wks" and 1 kg of *C. racemosa* "monoculture 4-6 wks", and 3 kg + 3 kg ("co-culture 0-3 wks") or 4 kg + 2 kg ("co-culture 4-6 wks") of *C. lentillifera* and *C. racemosa*, respectively. n = 3 trays for all treatments, mean ±SE.

Figure 3. Percentage of the harvestable biomass which is fronds in monocultures and co-cultures of *Caulerpa lentillifera* and *C. racemosa* (mean % +SE). The harvestable (above tray) portion of the biomass is the saleable portion.

Figure 4. Biomass properties of *Caulerpa lentillifera* and *C. racemosa* in monocultures and co-cultures. a) Density of fronds (mean # per cm +SE) for each treatment. b) Length of fronds (mean height in cm +SE).

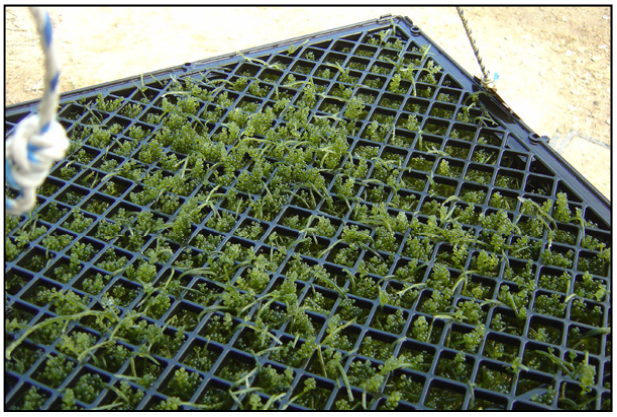
Figure 5. a) Total fatty and total polyunsaturated fatty acid content of *Caulerpa lentillifera* and *C. racemosa*. Data show means (+SE) for mg g^{-1} dry weight. b&c) ω-3 fatty acid content

related to frond size of *C. lentillifera* and *C. racemosa* of b) α- Linolenic acid (18:3), and, c) Eicosapentanoic acid (EPA, 20:5). Significant correlations shown ($p < 0.05$).

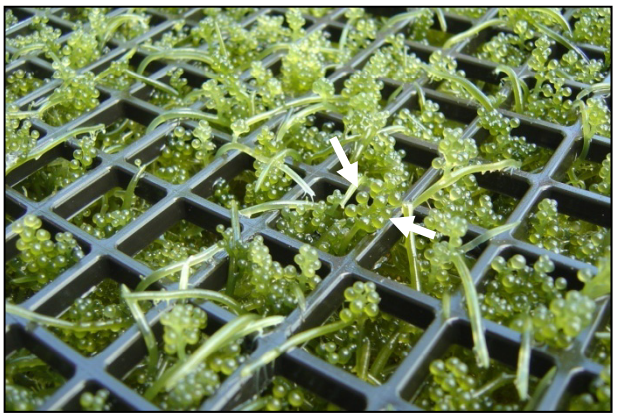
Figure 6. a) Primary pigment content of *Caulerpa lentillifera* and *C. racemosa*. Data show means (+SE) for mg g^{-1} dry weight. b&c) Pigment content related to frond size of *C. lentillifera* and *C. racemosa* of b) chlorophyll-b and c) β-carotene. Significant correlations shown ($p < 0.05$).

Figure 1.

a)



b)



c)

Figure 2.

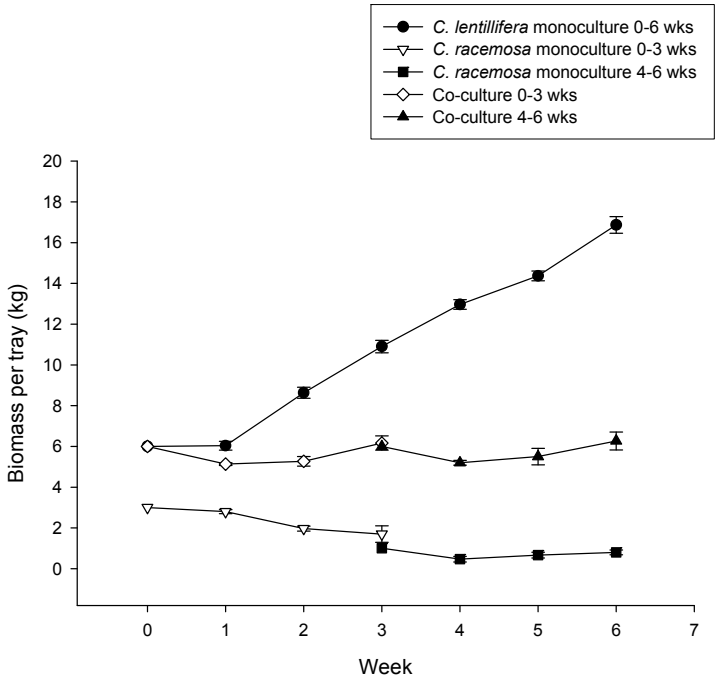

C. lentillifera monoculture 0-6 wks
C. racemosa monoculture 0-3 wks
C. racemosa monoculture 4-6 wks
Co-culture 0-3 wks
Co-culture 4-6 wks
Biomass per tray (kg)
Week
0
2
4
6
8
10
12
14
16
18
20
0
1
2
3
4
5
6
7

Figure 3.

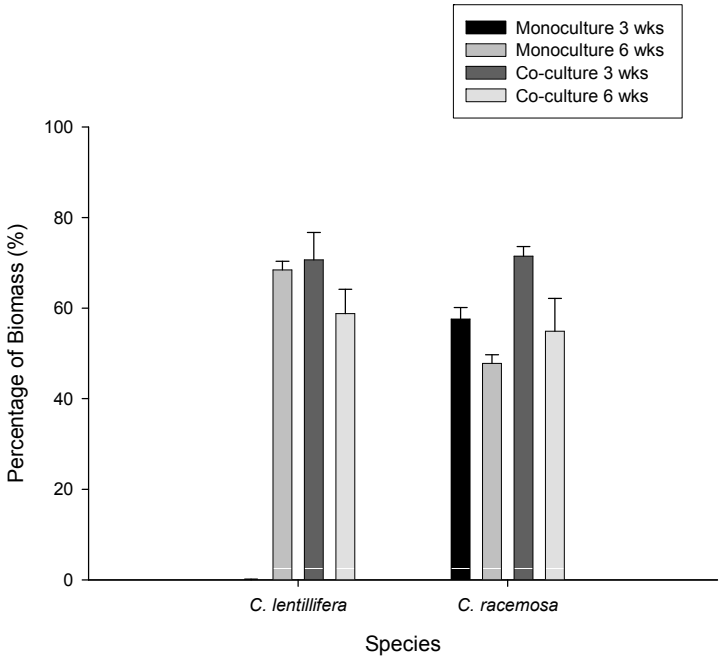

Monoculture 3 wks
Monoculture 6 wks
Co-culture 3 wks
Co-culture 6 wks
Percentage of Biomass (%)
100
80
60
40
20
0
C. lentillifera
C. racemosa
Species

Figure 4.

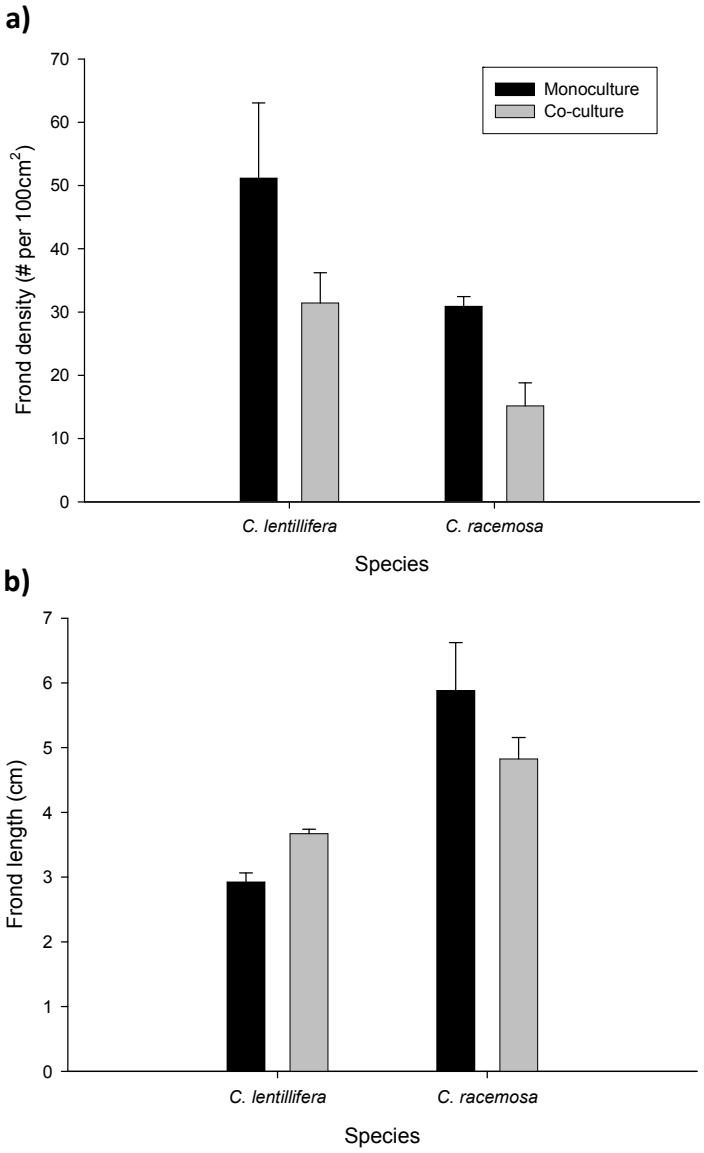

a)
Monoculture
Co-culture
Frond density (# per 100cm^2)
70
60
50
40
30
20
10
0
C. lentillifera
C. racemosa
Species
b)
Frond length (cm)
7
6
5
4
3
2
1
0
C. lentillifera
C. racemosa
Species

Figure 5

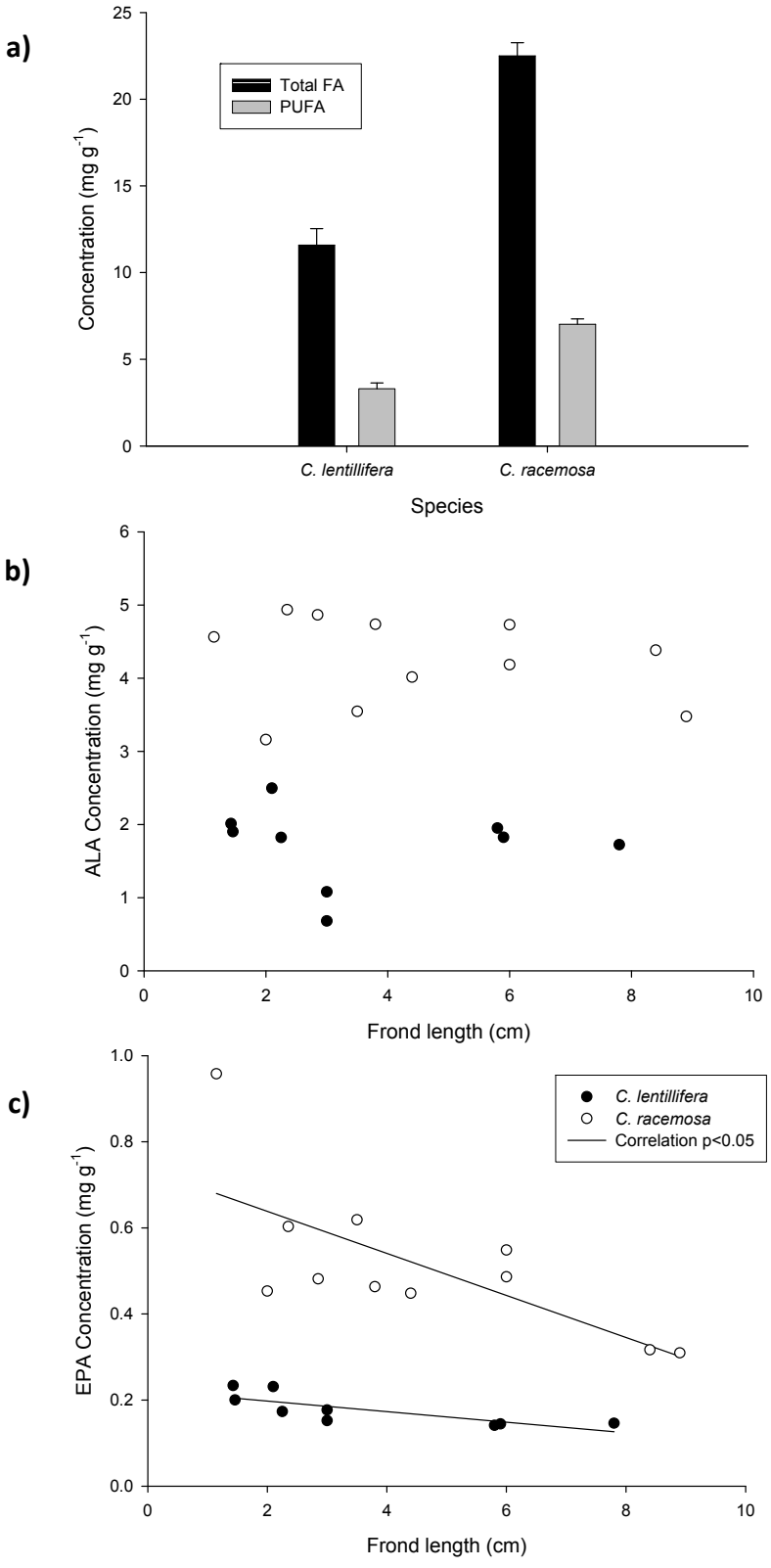
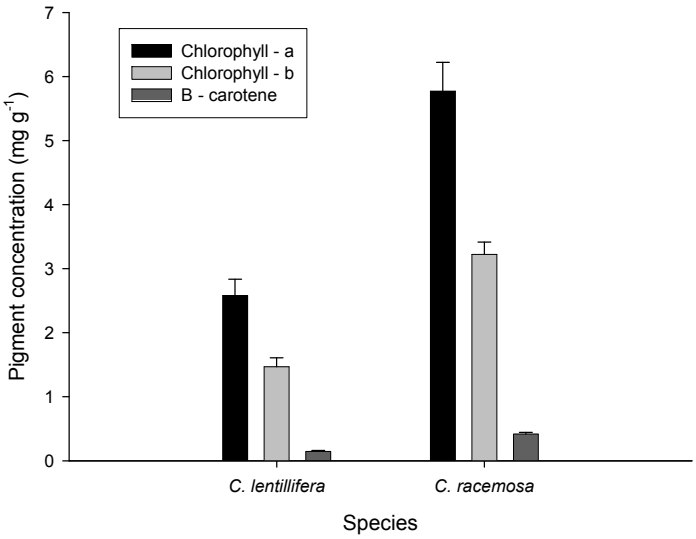
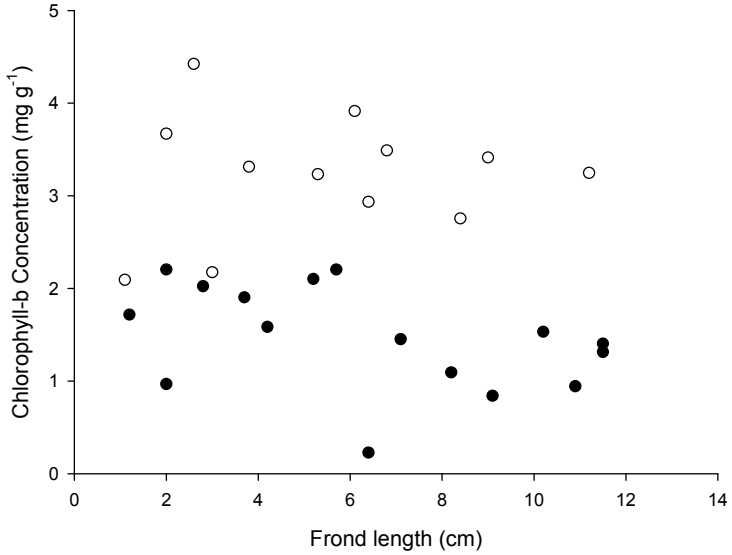

a)
Total FA
PUFA
Concentration (mg g-1)
C. lentillifera
C. racemosa
Species
b)
ALA Concentration (mg g-1)
Frond length (cm)
c)
C. lentillifera
C. racemosa
Correlation p<0.05
EPA Concentration (mg g-1)
Frond length (cm)

Figure 6

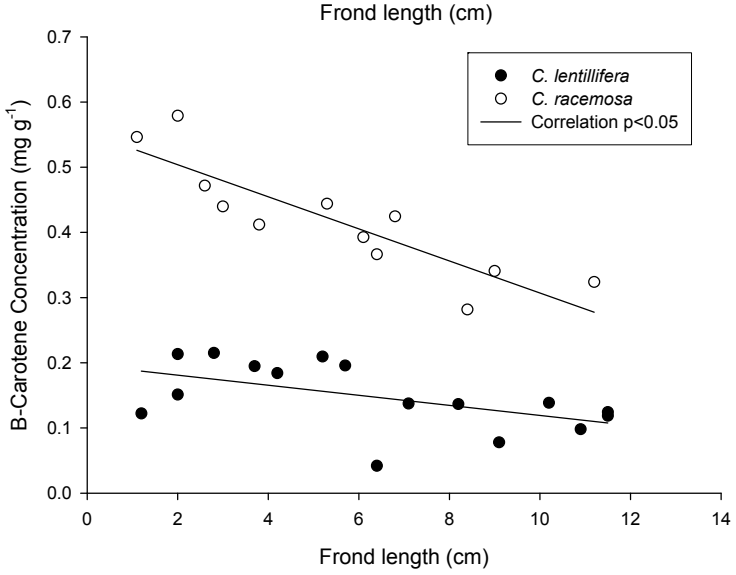
a)


Chlorophyll - a
Chlorophyll - b
B - carotene
Pigment concentration (mg g-1)
0
1
2
3
4
5
6
7
C. lentillifera
C. racemosa
Species

b)


Chlorophyll-b Concentration (mg g-1)
0
1
2
3
4
5
0
2
4
6
8
10
12
14
Frond length (cm)

c)


C. lentillifera
C. racemosa
Correlation p<0.05
B-Carotene Concentration (mg g-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
2
4
6
8
10
12
14
Frond length (cm)