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Salinity stress enhances protein content and amino acid profile in *Gracilaria cornea* (Rhodophyta)

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Abstract

Marine macroalga are frequently exposed to environmental stresses impairing their overall physiology and growth potential. Among these, *Gracilaria cornea* (Rhodophyta) is a valuable red seaweed rich in protein and polysaccharides. To investigate its physiological responses under controlled conditions, we cultivated *Gracilaria cornea* in an indoor culture system at three different salinity levels (30, 40 and 50 ppt), employing continuous aeration, blue and white LED illumination (12:12 light:dark cycle), and exogenous addition of nitrogen and phosphorus. Physiological changes associated with protein content accumulation and amino acid composition were determined using in-situ reflectance spectroscopy (VIS-NIR range 560-674 nm), AI algorithm and GC-MS analysis. We developed novel tools to accurately predict amino acid composition and total protein yield, identified the environmental factors inducing trait accumulation and determined the optimal harvesting day. Hypersaline stress and cultivation day significantly influenced

protein content with optimal protein content (> 35% dry weight) achieved on day 14. This peak was not correlated with the specific growth rate (SGR), indicating SGR may not reliably indicate protein yield in this context. The dry weight to fresh weight ratio (DW:FW) was higher under hypersaline conditions, leading to a greater dried biomass and higher protein content, despite a reduced overall growth rate. Protein content was maximal under high ambient pH and high salinity. Day 14 was optimal for the highest yield of essential amino acids (EAA), exceeding 40% of the total amino acids. The algorithmic model accurately predicted specific amino acid proportions.

Keywords: *Gracilaria*; seaweed; protein concentration; amino acid composition; salinity; reflectance spectroscopy; prediction

1. Introduction

Today's world is increasingly looking to the oceans, and in particular to marine seaweed, as a sustainable source of protein for food security [1]. These species are nutrient-rich and possess small environmental footprints, but a major drawback for their potential use in the global industrial market is the lower protein concentration of marine macroalgae compared to terrestrial agricultural products [2]. This is primarily due to the high water content in alga tissues which can reach 74-94% of the total weight [3]. However, the total protein content expressed as a percentage of the dry weight was reported to be as high as 26% and 47% of green and red macroalgae, respectively [4]. The proportion of protein content and its nutritional profile in terms of amino acid composition are key factors for applications in the food industry [5]. The amino acid composition of marine macroalgae, especially Rhodophyta, is rich and usually contains all the essential amino acids [6].

The enhancement of protein yield from marine macroalgae while maintaining its nutritional profile has been extensively studied [4, 7]. Such studies include exploration of the biophysiological mechanisms involved in seaweed tolerance to euryhaline stressors [8, 9] and their effect on the propagation of traits-of-interest. Among the species that have been commercialized worldwide, edible species of

the red seaweed *Gracilaria* (Rhodophyta) are of particular interest for their high quality and high yield in proportion to their dry weight, of potentially edible protein compounds [10] and essential amino acids ([5] having bioactive and functional properties [11, 12, 13]. Species of the genus *Gracilaria* grow seasonally well under diverse seawater environments [14], such as the Eastern Mediterranean Sea, and in SE Asia [15], where there is extensive aquaculture production for the agar and agarose markets used in the pharmaceutical and biotechnology industries [16, 17]. Previous studies have shown that this euryhaline species [18, 19] can acclimate and inhabit intertidal zones, in a wide range of salinity levels (from 0.5 to 60 ppt), [13], seawater temperatures (from 12 °C to 32 °C), [20, 21] and nutrient availability [22], as well as deep water with low irradiance intensities [23, 24]. The response to changes in salinity involves water fluxes into and out of seaweed cells. This biological mechanism, which enables intertidal species to rapidly adjust to salinity stress [25,26, 27] Donadio et al. 2025), encompasses the expression of a wide range of metabolites [18, 28], including amino acids, carbohydrates, proteins, enzymes, chromophores like phycobiliproteins, and antioxidants [29, 8, 30]. Compounds induced by salinity stress, are also associated with phenotypic alterations including changes in the algal chromophore and in some cases overall biomass shrinkage [31, 32]. Under conditions of hyper- or hypo-salinity, the red seaweed uses photosynthesis activity, inorganic ions and organic osmolytes to regulate an osmotic gradient. For instance, during cell division and elongation [33], algae accumulate water to support the metabolic process, but exposure to hyper-or hypo-salinity conditions that are usually involved in cell differentiation inhibition can occur: this may reduce growth rate [34]. The (eco)physiological process associated with growth rate has been poorly addressed in seaweed studies to date.

Previous studies have extensively examined the impact of salinity stress and desiccation on the ecophysiological performance of seaweeds [35, 36]. Biomass production of macroalgae in response to hypo- and hyper-saline conditions [28, 37] is reflected in photosynthesis inhibition, respiration, and impaired metabolic responses [35, 18, 8, 30]. Salinity stress and desiccation constitute two different forms of water deprivation in seaweed tissues. The interactions between salinity stress and other abiotic factors such as temperature, ambient pH, irradiance, and nutrient availability on phenotype alteration and nutritional properties have also

been previously evaluated [38, 39]. Interestingly, the process of acclimation to salinity stress was not observed to hamper the nutrition profile in terms of amino acid composition and overall protein yield [8, 40]. However, precise tools for manipulating, monitoring, controlling, and predicting the induced response of protein propagation and amino acid composition to salinity stress are presently unavailable.

In this research, we studied the potential of manipulating the salinity response and acclimation mechanisms of *Gracilaria cornea* under abiotic stress by exposure to hyper-and hyposaline culture conditions. We also analyzed the effect of various conditions of salinity on protein accumulation and amino acid composition in *Gracilaria cornea* using predictive models that support decision-making (DSS), previously developed [17, 41]. Optimized cultivation protocols harness the natural adaptive responses of red algae to a variety of environmental stressors. DSS models enable precise control of cultivation conditions to ensure high-quality, protein-rich biomass. Predictive models estimate protein yield and amino acid composition throughout the cultivation cycle. Together, these capabilities foster precision in production, promote efficient resource use, support regulatory compliance, reduce risk, and, in some cases, prevent economic losses.

2. Materials and Methods

2.1 Sample collection and preparation

Specimens of the edible red seaweed *Gracilaria cornea* were collected from a rocky intertidal zone at Dor-Habonim Beach, Israel (N 34°55´32" E 32°02´39"), in the easternmost Mediterranean Sea. In Israel, seaweeds do not require specific permits for collection by the local Ministry of Environmental Protection. Vouchers of these specimens are deposited at the public available seaweed collection at IOLR (https://www.seaweedherbarium.com/), and were identified by Á. Israel. The easternmost Mediterranean Sea is recognized by being largely oligotrophic with significantly lower nutrient concentrations compared to the western basin [42, 43]. Coastal waters at Dor-Habonim Beach exhibit seasonal variability in temperature (ranging from ~16 °C in winter to ~30 °C in summer) and stable salinity (typically 38–39 ppt year around), resulting from both regional climatic influences and localized evaporation-precipitation dynamics [44]. Upon collection, *Gracilaria cornea* was acclimated for two weeks in a 700 L PVC tank supplied with

running seawater and constant aeration in an outdoor cultivation system at the Israel Oceanographic & Limnological Research (IOLR) in Haifa, Israel. Samples from this acclimated seaweed stock were then brought to an indoor setup consisting of nine, 16-L aquaria (n=9) also supplied with inflowing seawater and continuous aeration. Specimens were cultured at natural seawater salinity of 40 ppt (parts per thousand), under a mix of blue and white LED illumination, with a 12:12 light:dark cycle at an average intensity of 22.4 ± 4.0 µmol photons m^{-2} s^{-1} and average water temperature of 24.0 ± 0.45 °C. The seaweed specimens were acclimatized to these new indoor conditions for an additional two weeks prior to the initiation of the experiments.

2.2 Experimental setup

The indoor experimental setup consisted of nine 16 L aquaria ranged in block design [45], filled with seawater and randomly assigned to three levels of salinity (30, 40 and 50 ppt, **Fig. 1**). The first three aquaria were supplied with seawater reduced to 30 ppt by adding freshwater. The second group of three aquaria were supplied with regular seawater at a salinity of 40 ppt designed to mimic the natural habitat conditions of *Gracilaria cornea* at the intertidal zone in the Israeli Mediterranean Sea [46]. This group served as the control. The third group was supplied with seawater supplemented with natural dry salt (Red Sea, 8 dKH, Israel) composed of salt mix: magnesium (Mg^{2+}, 43%); sulphate (SO_4^{2-}, 30%), calcium (Ca^{2+}, 14%), potassium (K^{+}, 13%) and trace elements (0.46%). The salinity level was adjusted to hypersaline conditions of 50 ppt.

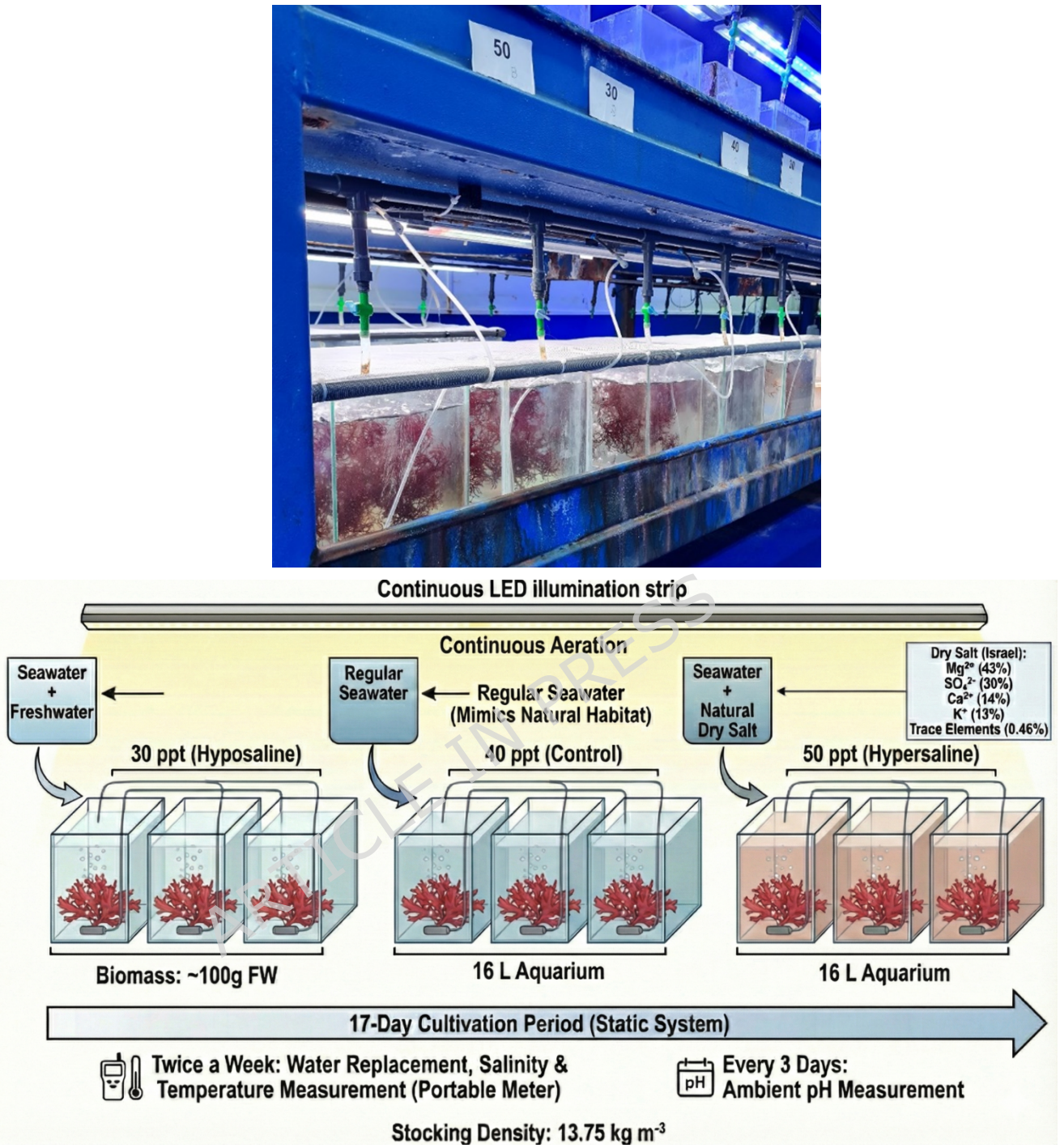


Figure 1. The indoor setup designed for the cultivation of *Gracilaria cornea* in three salinity regimes.

Following acclimation, approximately 100 g fresh weight (FW) *G. cornea* were placed in each aquarium (n=9) at a unified stocking density of 13.75 kg m^{-3} and grown for 17 consecutive days. Culture media were continuously aerated [22], and inflow of seawater was stopped during the cultivation period. The culture media were replaced twice a week and adjusted to the appropriate hypo- or hyper-

salinity level as described above. At each water replenishment, the salinity level (ppt) and seawater temperature (C°) were measured manually by submerging a portable conductivity meter (Thermo Scientific™ Orion Star™ A222, Thermo Scientific™, USA) in the cultivation medium. LED illumination was provided from an overhead strip, and the intensity was identical to conditions of the acclimation period. Ambient pH was measured by sampling the culture water from each aquarium every three days during the cultivation period.

2.3 Nutrient supplementation regime

The nutrient supplementation regime was determined according to preliminary results that evaluated the effective nitrogen and phosphate supplementation dosage (using a pulse-feeding approach, [47] to support maximal protein yield of *Gracilaria*. In accordance with these preliminary results, high fertilization addition (HF) of ammonium (2.0 millimole, added as NH_4Cl) and phosphorus (0.2 millimole, added as PO_4^3) was applied at the beginning of every week (at start point[T0], day 6, and day 13), thereafter moderate levels of fertilization (MF) addition of ammonium (1.0 mM) and phosphorus (0.1 mM) were applied in the middle of each week (days 2, 9 and 16).

2.4 Determination of protein content

To determine protein concentration in-situ we used the model proposed by [17]. The predictive models that enable determination of the protein content are based on spectroscopy measurements on the alga thallus in the VIS-NIR range (400-1000 nm) and an artificial neural network (ANN) algorithm.

Spectral features were obtained outdoors on a fully sunny day via a field spectrometer (USB4000, Ocean Optics Inc, Dunedin, FL, USA) calibrated with a Spectralon plate (Labsphere Inc., North Sutton, NH, USA). Diffuse reflectance measurements were conducted using a bare fiber optic probe, moving point by point above the seaweed thalli, to capture as much chromophore diversity as possible [48]. Spectral data were collected in the 400-1000 nm VIS-NIR range, with resolution of 0.5 nm and accuracy of 1 nm, with 10–15 spectra repetitions for each sample [49].

The algorithm uses information on seaweed phenotype chromophores obtained from in-situ spectroscopy measurements and an ANN to estimate protein concentration (% DW). The ANN algorithm is trained to convert phenotypic spectral features and pigment intensity of *G. cornea* thallus acquired in the visible near infrared (VIS-NIR) spectrum (560-674 nm) into accurate protein content predictions when compared to analytical results of nitrogen content. Protein content was assessed in vivo by field spectroscopy and a trained ANN algorithm, normalized and processed using the Kubelka-Munk approach [50] for pigment intensity calculation [17].

Our previous results also validated the earlier finding by [51] that the most accurate nitrogen to protein conversion factor for Rhodophyta is 5.0. Here, randomly selected *G. cornea* replicates from each aquarium were harvested 24 h after pulse supplementation of ammonia and phosphorus. The replicates from each salinity treatment were analyzed through remote sensing and non-destructive reflectance measurements for protein content determination.

In addition, we employed a decision support model developed in our previous study [41] to identify key biotic and abiotic factors influencing protein yield and to determine the optimal harvesting day.

2.5 Determination of moisture content (MC) and FW:DW ratio

Moisture content (MC) was analyzed to explore possible alteration in organic matter and morphology variabilities as a response to hyper and hypo-salinity conditions [8]. To determine FW:DW ratios [52] the randomly selected and harvested fresh *G. cornea* replicates were washed in tap water, weighed for FW determination, and then weighed for DW after drying in an oven at 60 °C for 48 hours. For ash content we used previous reported results [53]. Moisture content was determined using the following equation [54].

$$\text{MC content (\%)} = \frac{(B_f - B_d)}{B_f} \times (100\% - \text{ash\%}) \quad (2)$$

where B_f and B_d (in grams) are the initial wet weight and oven dry weight, respectively, of the *G. cornea* replicates, and ash is the proportional ash content of the dried biomass.

2.6 Determination of amino acid composition

To evaluate the effect of salinity level on amino acid (AA) composition and content, the oven-dried replicates of *G. cornea* from each culture regime, were finely milled and analyzed using GC-MS (Thermo Scientific Trace 1310 GC and Thermo Scientific ISQ LT GC-MS [both Thermo Fisher Scientific, Waltham, MA USA]).

2.6.1 Algae tissue hydrolysate

Approximately 7 mg of lyophilized alga tissues were hydrolyzed in 0.5 ml HCL 6N at 150 °C for 70 min under a nitrogen atmosphere in a 4 mL glass vial with a PTFE cap [55]. Samples were cooled to room temperature and the HCl was evaporated under a gentle stream of nitrogen.

2.6.2. Sample derivatization

For derivatization we used the protocol of [56] based on esterification and acylation. Briefly, each sample underwent an acid-catalyzed esterification using isopropanol and acetyl chloride (5:1) followed by acylation with trifluoroacetic anhydride and dichloromethane (1:1).

2.6.3. GC-MS protocol

Triplicates of 2 µl of each sample and amino acid calibration standard were injected in split mode (1:15) at 250 °C. The amino acids were separated on a Zebron ZB-5MSplus column (60 m, 0.32 mm, and 1 µm) on a Thermo Scientific Trace 1300 GC (Thermo Fisher, Waltham, MA USA). The gas chromatography (GC) conditions were set to optimize peak separation for the desired amino acids: initial temperature 90 °C ramped to 300 °C at 6.5 °C per min and then ramped to 320 °C at 20 °C per min and held for 2.5 min. For amino acid analysis we used Thermo Scientific Xcalibur software. Calibration curves were constructed for 15 amino-acids: alanine, glycine, threonine, serine, valine, leucine, isoleucine, proline, aspartic acid, methionine, glutamic acid, phenylalanine, lysine, tyrosine and histidine. The measured values of tryptophane, cysteine and arginine were

excluded from the model since these amino acids were either destroyed during derivatization or have not been detected. Data on amino acid composition was reported as percentages of the specific amino acid out of the total AA quantity [12]. The accuracy and precision of the analysis were confirmed through the use of calibration standards.

2.7 Statistical analysis

Linear mixed models restricted to maximum likelihood [57] were used to analyze separately the responses of protein propagation (% DW) and amino acid composition (% of total AA) induced in *G. cornea* by culturing in hyper- or hypo-salinity conditions. The input data for protein determination was obtained from in-situ spectral measurements of alga thalli phenotype collected in the VIS-NIR range of the electromagnetic spectrum (560-680 nm) and the ANN algorithm. Determination of AA composition and content was obtained from CG-MS analysis. Each of the nine aquaria (blocks) is considered a complete randomized unit. The day number (Di) with six levels was defined as a nominal classification of fixed factors to account for the nonlinear protein expression response of *G. cornea* under abiotic stress [17, 41, 58] and for predictive performance improvement of both protein content and AA composition. Other variables such as salinity (Si) with three levels, ambient pH, temperature, the ratio between dry weight to fresh weight (DW:FW) and their interaction with salinity level and day number were treated as fixed effects as well. Statistical models were fitted using the Generalized Linear Mixed Model (GLIMMIX) procedure of SAS software, version 9.4 (SAS inst. Inc. Cary NC, USA). All statistical analysis were performed in triplicates and Bonferroni grouping for least square means ($\alpha = 0.05$) was applied for post hoc comparisons. Results were considered significant at $P \leq 0.05$.

The performances of the model in predicting specific AA concentration (sAA) were assessed in terms of the regression coefficient R^2 and the root mean square error (RMSE)

$$RMSE = \sqrt{\sum_{i=1}^{n} \frac{(\hat{y}_i - y_i)^2}{n}} \quad (3)$$

where $\hat{y}_i$ and y_i are the predicted and measured values of sAA concentration and n is the number of samples.

3. Results

3.1. Abiotic culture conditions

Abiotic details are shown in **Table 1**. Average ambient temperatures in the aquariums varied from 23.18 ± 0.52 to 25.22 ± 0.83 °C. These fluctuations were influenced by indoor conditions, tap water temperature and the state of the seawater conditions during the replenishment of the cultivation medium, which were affected by both outdoor temperatures and solar irradiance. The average light intensity, which was influenced by aeriated water turbulence and the biomass density accumulation of *G. cornea*, was lower under control conditions (40 ppt) compared to hypo and hyper-saline conditions (19.03 ± 2.96, 23.77 ± 5.03 and 24.99 ± 4.44 µmol photons m^{-2} s^{-1} respectively). These results suggest a potential decrease in growth rate and reduced biomass density accumulation under saline stress. On the other hand, the differences in biomass density can be also explained by osmotic adjustment [40] as an acclimation response to salinity stress which is involved with overall thallus shrinkage and reduction of the biomass fresh weight. Differences in the thallus rigidity and chromophore pigmentation, and significant decrease in growth rate under hyper-and-hypo-saline conditions were also identified by [22]; and [59].

The ambient pH level remained stable under hyper-saline conditions (50 ppt) throughout the trial period, averaging 8.07. Under 30 ppt conditions ambient pH remained relatively stable, averaging 8.03, but it decreased to 7.82 at day 10. Under control conditions (40 ppt) the pH level was less stable, fluctuating up and down between 7.94 and 8.04 (**Table *1***).

Table 1. Abiotic culture conditions (pH, temp, irradiance) and biotic measurements (moisture content -MC (%), dry to fresh weight ratio - DW:FW and protein content -% DW): For specific day of cultivation (1-17) under three salinity regimes (30, 40 and 50 ppt).

G. cornea

Salinity (ppt)	Day	pH	Temp (°C)	Irradiance (µmol photons m^{-2} s^{-1})	MC (%)	DW:FW	Protein (% DW)

30	1	8.07 ± 0.00	25.76 ± 0.46	17 ± 0.0	79.05 ± 0.99	0.26	14.54 ± 0.56 **G**
	3	8.06 ± 0.01	24.03 ± 0.16		79.85 ± 0.15	0.25	21.46 ± 0.25 **F**
	7	8.06 ± 0.01	26.56 ± 1.19	22.33 ± 4.18	80.78 ± 0.42	0.24	27.92 ± 2.05 **D,E,C**
	10	7.82 ± 0.04	25.36 ± 1.05	21.0 ± 1.41	79.14 ± 1.46	0.26	27.74 ± 0.98 **B,D,A,C**
	14	8.05 ± 0.01	24.4 ± 0.14	25.0 ± 13.58	77.81 ± 2.65	0.29	29.89 ± 1.04 **B,D,C**
	17	8.12 ± 0.01		33.5 ± 10.30	80.03 ± 0.44	0.25	28.57 ± 0.98 **D,E,C**
40	1	8.0 ± 0.00	24.0 ± 0.42	17 ± 0.0	78.40 ± 0.30	0.28	16.13 ± 0.65 **F,G**
	3	7.9 ± 0.03	22.9 ± 0.08		78.70 ± 0.21	0.27	19.75 ± 0.49 **F,E**
	7	7.9 ± 0.03	23.6 ± 0.64	19.33 ± 2.05	78.68 ± 0.07	0.27	27.17 ± 1.12 **B,D,C**
	10	8.04 ± 0.01	22.73 ± 0.09	18.33 ± 1.24	78.45 ± 0.42	0.27	27.24 ± 0.90 **D,E,C**
	14	7.94 ± 0.01	22.7 ± 0.08	16.0 ± 2.16	78.82 ± 0.42	0.27	31.21 ± 0.29 **B,A,C**
	17	7.96 ± 0.02		24.5 ± 9.75	78.74 ± 0.03	0.27	32.59 ± 1.82 **B,A,C**
50	1	8.07 ± 0.01	24.0 ± 0.35	17 ± 0.0	77.77 ± 0.11	0.29	18.21 ± 0.77 **F,G**
	3	8.07 ± 0.02	23.76 ± 0.67		77.43 ± 0.61	0.29	23.31 ± 0.61 **F,D,E**
	7	8.09 ± 0.01	23.16 ± 0.04	21.0 ± 5.09	77.41 ± 0.30	0.29	28.52 ± 0.19 **D,E,C**
	10	8.07 ± 0.01	23.0 ± 0.14	26.66 ± 7.84	77.09 ± 0.44	0.30	28.64 ± 1.14 **D,C**
	14	8.04 ± 0.01	24.6 ± 1.01	28.66 ± 6.18	76.66 ± 0.79	0.30	35.61 ± 2.8 **A***
	17	8.06 ± 0.01		28.33 ± 11.78	76.85 ± 0.66	0.30	35.0 ± 1.89 **B,A**

 Different letters indicate significant differences. Asterix indicates the highest result.

3.2 The effect of salinity level on MC and DW:FW ratio

The ash content of raw biomass was assumed to be 12.7% [53]. The moisture content (MC) of *G. cornea* specimens under natural saline conditions (40 ppt) remained stable, averaging 78.63 ± 0.24%, from day 1 to day 17, demonstrating dry weight to fresh weight ratio (DW:FW) of 0.27 (**Table *1***). However, under hyposaline conditions (30 ppt), the MC fluctuated between 80.78 ± 0.42% to 77.81 ± 2.65% thus yielding a DW:FW ratio of 0.24 on day 7, which increased to 0.29 on day 14. Under hyper-saline conditions, replicates of *G. cornea* responded rapidly by retaining low MC between day 1 and day 10, averaging 77.43 ± 0.36%.

The MC continues to decrease between day 14 and day 17 reaching 76.75 ± 0.72%. The highest ratio of dried ash-free biomass relative to fresh weight was obtained under high saline conditions, reaching 0.30.

3.3 The effect of salinity level on protein content accumulation

Total protein content of *G. cornea* as determined by spectral measurements and ANN algorithm (**Table *1***), was significantly affected both by salinity level ($P = 0.0028$) and the day of cultivation ($P < 0.001$,). Additionally, the combined effect of day and salinity level on protein manifestation in the algae was significant as well ($P = 0.0077$). Hyper-salinity conditions (50 ppt) consistently induced a higher level of protein manifestation response compared to specimens cultivated under both natural salinity and low salinity conditions (40 and 30 ppt respectively, post hoc Bonferonni grouping, $\alpha = 0.05$). The highest protein accumulation of 35.6 ± 2.83% (DW), was observed on day 14 in specimens cultivated under hyper-salinity conditions (50 ppt) which was 12.36% higher in comparison to protein level obtained from the control culture. This physiological response differs significantly from the response observed under 30 ppt conditions in which the accumulated protein content reached 29.89 ± 1.04% (**Table *1***; ***Fig. 2***). Interestingly, the experimental results obtained from controlled indoor cultivation conditions, supplied with the pulse feeding nutrient regime, revealed that the algae were acclimated rapidly to the varying salinity level by exhibiting different protein levels already at day 1 reaching 14.54 ± 0.57%, 16.13 ± 0.65%, 18.21 ± 0.77% under hypo-control and hyper salinities, respectively. Protein accumulation gradually increased at all salinities up to day 7, similarly to the results obtained by [22]. Overall, the algae response to hyper-salinity (50 ppt) differed significantly from the other salinities with regard to its protein synthesis. The GLIMMIX model also revealed that the protein level was positively and significantly affected by the ambient pH ($P = 0.00362$) for all the values of salinity. In the hyposaline case (30 ppt), the ambient pH varied between 7.82 to 8.12 with the highest protein manifestation response of 29.89 ± 1.04% (DW) being obtained on day 14. For the control (40 ppt) the pH range was 7.94 to 8.04 with the highest protein content of 32.59 ± 1.82% being obtained on day 17, while

under hypersaline conditions (50 ppt) the pH varied from 8.04 to 8.09 with the highest protein content of 35.61 ± 2.83% being obtained on day 14 (**Table *1, Fig. 3***). The parameters that exhibited significant effects are detailed in **Table 2**. Neither temperature nor ambient nitrogen and phosphorus significantly affected the total protein content. These findings are only partially consistent with previous studies [38, 22]. Contrary to [38], in our study the salinity significantly affected the protein content, suggesting an efficient mechanism of acclimation for algae in response to salinity stress.

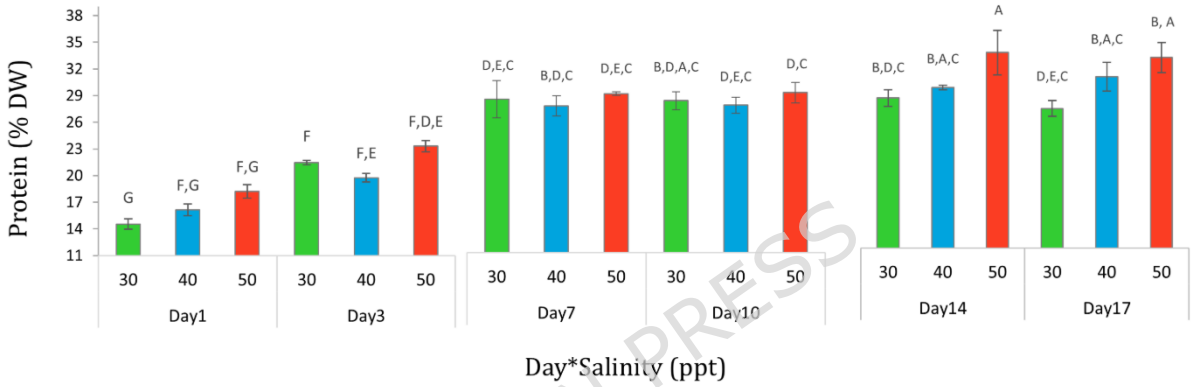


Figure 2. Protein content (% DW) of *G. cornea* on days 1-17 cultured at three levels of salinity (30, 40, 50 ppt). Data represent means of triplicates ± SD. Bars with different letters indicate significant differences.

Table 2. The significant affected fixed factors included in the GLIMMIX for prediction of protein content accumulation (% DW); and AA composition including total EAA (%); total NEAA (%), the EAA/NEAA ratio and concentration of specific amino acid (sAA, %). Asterix indicates interaction between two factors.

Fixed Effect	Num DF	Den DF	F Value	P value
Protein level				
Day	5	28	174.99	<.0001
Salinity	2	28	7.32	0.0028
Day*Salinity	10	28	3.17	0.0077
pH	1	28	4.84	0.0362
Total EAA				
Day	4	31	7.47	0.0002
Temp	1	31	4.38	0.0445
DW:FW	1	34	8.39	0.0066
DW:FW*Day	5	34	2.87	0.0289

Total NEAA				
Day	5	40	9.23	<.0001
EAA/NEAA ratio				
Day	5	40	4.92	0.0013
Specific AA				
sAA	14	641	1058.76	<.0001
Day	5	641	10.08	<.0001
Day*sAA	70	641	5.47	<.0001
Salinity*sAA	28	641	5.43	<.0001

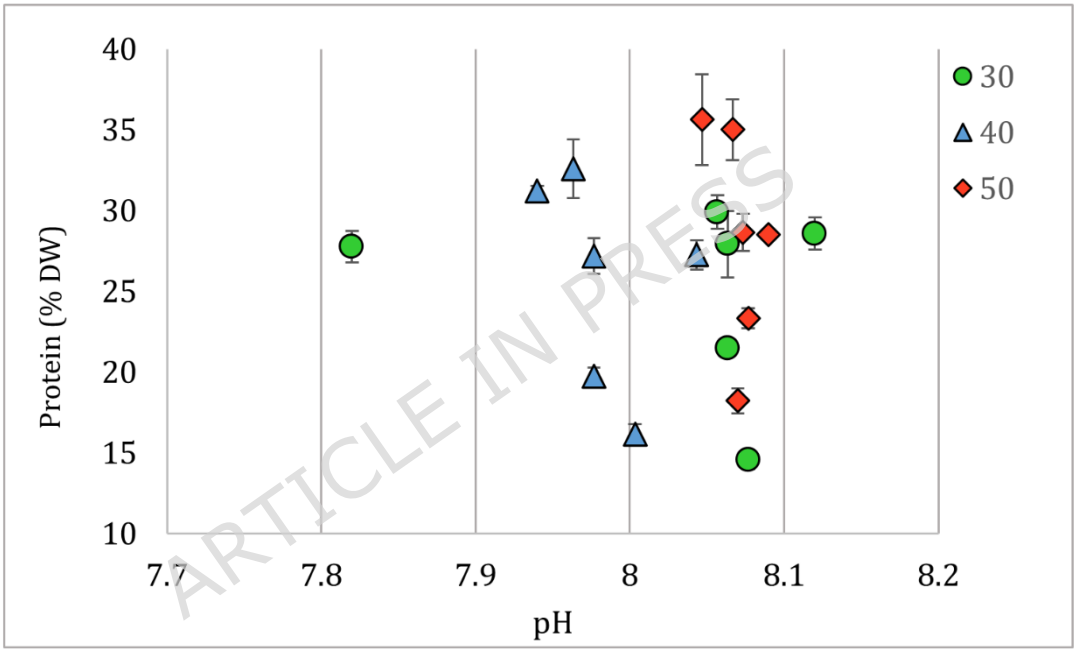


Figure 3. The effect of pH and salinity levels on protein accumulation

3.4 Effect of salinity level on amino acid composition

GC-MS analysis of *G. cornea* revealed the presence of 15 amino acids, including eight essential amino acids (EAA) i.e. leucine, isoleucine, lysine, methionine, phenylalanine, threonine, histidine and valine. EAA must be acquired through diet since the human body cannot synthesize them [60]. Use of GLIMMIX showed that total EAA was significantly affected by the temperature (p = 0.0445), the day of cultivation (p = 0.0373), the DW:FW ratio (p = 0.0052) and the interaction between these two parameters (p = 0.0151) (**Table 2**). It

appears that the effect of salinity level on EAA accumulation was slightly above significance ($p = 0.0504$) which can be an indication of the resilience of this species to salinity stress. Interestingly, on day 1 ($p = 0.0234$) and day 10 ($p < 0.01$), for all salinity regimes, as the DW:FW ratio increased the proportion of EAA decreased.

For all salinities, the mean ratio of EAA to the total AA increased by 18% from 35.32 ± 1.83% on day 1 to 41.67 ± 1.74% on day 17. Among the EAA, valine (8.73 ± 0.60% in 30 ppt, 7.86 ± 0.29% in 40 ppt, 8.34 ± 0.65% in 50 ppt) was the most abundant EAA (in terms of % of all AA by weight, **Fig. 4**) followed by leucine (7.39 ± 0.50%, 6.66 ± 0.29%, 6.78 ± 0.42%) and isoleucine (5.98 ± 0.43%, 5.33 ± 0.19%, 5.53 ± 0.44%) under hypo-saline, control and hyper-saline conditions, respectively. Valine, as well as leucine and isoleucine, are reported to help stimulate muscle growth and regeneration, regulate blood sugar levels and wound healing, produce growth hormones, play an important role in immune function and are involved in energy production [61]. Seven non-essential amino acids (NEAA) were detected as well, including alanine, glycine, serine, proline, tyrosine, glutamic acid and aspartic acid. The last two NEAA were the most abundant (11.24 ± 0.79% and 10.92 ± 0.73%, respectively). The NEAA levels were significantly influenced by the day of cultivation ($p < 0.001$), peaking at 57.25% on day 17. Interestingly, the pattern of EAA to NEAA ratio in *G. cornea* remained stable under all salinity regimes, with a significant decrease observed on day 10 ($p=0.0003$). The lowest ratio (0.74:1) was observed under hypo-saline conditions, while both the control and hyper-saline regimes exhibited a ratio of 0.76:1 (**Fig. 4**). These results are consistent with other *Gracilaria* species, which exhibit EAA/NEAA ratios ranging from 0.67 in *G. vermiculophylla* [5] to 1.61 in *G. Changii* [62]. Aspartic acid and glutamic acid constituted, as in many other species of seaweed [63], the largest proportion of the total AA, in all the salinity regimes, 10.45 ± 0.65% and 11.70 ± 0.83% respectively

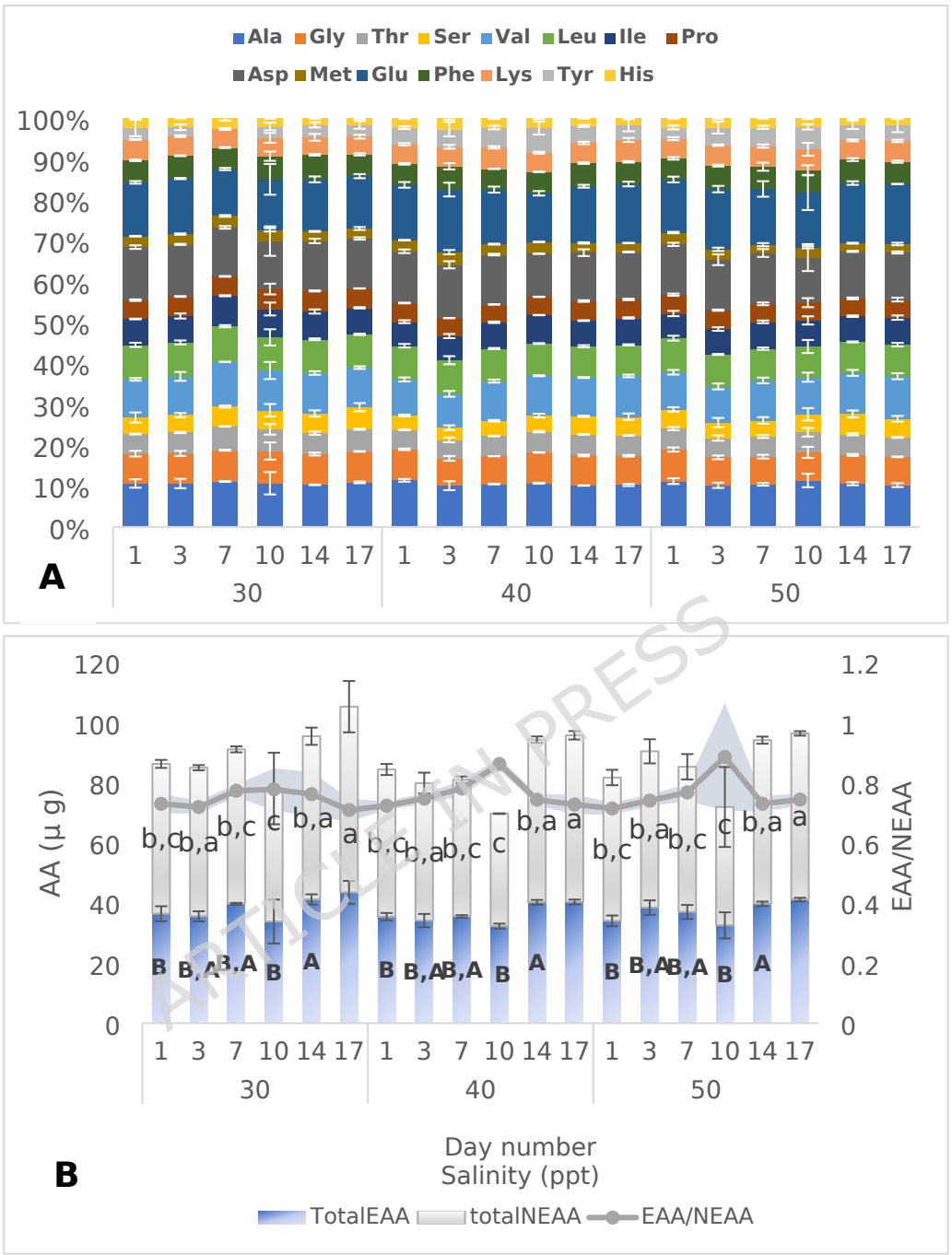
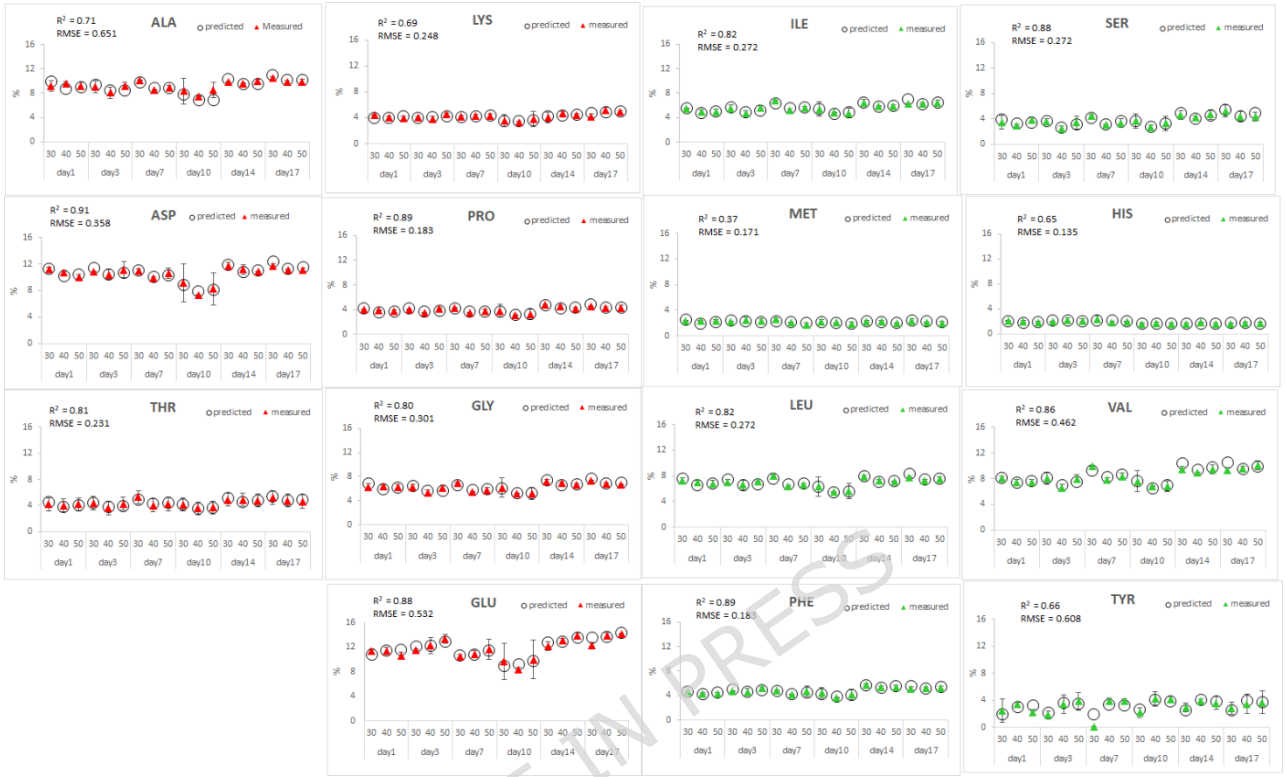


Figure 4. (A) Proportion of 15 amino acid (%) obtained from *G. cornea* specimens cultured under different salinity regimes, for specific day of cultivation: (B)ratio of total EAA to NEAA, and total AA(μg) Error bars represent standard error of triplicates. Shaded area in (B) represent minimum and maximum values of EAA/NEAA ratio. Results with different letters are differ significantly.

3.5 The model performance in predicting the content of specific AA

The day of cultivation interacted with salinity level both significantly affected the concentration of specific amino acid (sAA) as determined from the GLIMMIX model (**Table *2***). The model has been able to identify not only the ambient



conditions with significant effect induced by the alga responses, but also to predict reasonably well the concentration of all 15 AA (**Fig. 5**).

Figure 5. Accuracy of prediction (indicated by the black rings) for the proportion of sAA (%) biosynthesized in *G. cornea* according to salinity regime and day of cultivation. Values obtained from GC-MS analysis and represent means of triplicates ± SD. EAA and NEAA are represented by green and red colours, respectively.

For instance, the NEAA glutamic acid that plays significant roles in nutrition, signaling and metabolism [64], reached its highest level of 13.85% on day 17 under the hyper-salinity regime, and this peak significantly differs (Bonferroni grouping, $\alpha = 0.05$) from all the other days of cultivation and salinity regimes (**Fig. 4**). In contrast, the EAA valine, which was insignificantly affected by the salinity level, also reached its highest level on day 17 (9.96%). However, this value was only slightly above the level of valine obtained on day 14 (9.28%), which indicated that there was no significant change in the valine value as a function of cultivation day. Some of the amino acids were found to be significantly affected

as the ambient temperature increased through the period of cultivation (**Table 3**). For instance, as the temperature of the cultivation medium increased from 22 °C to 28 °C, the content of glycine, threonine, serine, valine, leucine, isoleucine, proline, aspartic acid and phenylalanine all reached peaks on day 14 regardless of the salinity level. As reported in previous studies, marine seaweed synthesizes sAA under environmental stress. For instance, the yield of glutamic acid, which plays a vital role in nitrogen metabolism and chlorophyll biosynthesis for cell repair [65, 66, 67] increased under high-temperature conditions in *G. cornea.* This was also found for proline and arginine. The same response has been obtained for other marine seaweeds such as *Ulva rigida* [68] and *Pyropia haitanensis* [69].

Table 3. The effect of ambient temperature (22 °C and 28 °C) over the period of cultivation (1-14 days) on the biosynthesis of sAA (%). Numbers with different letters are significantly different, as obtained by a post hoc Bonferroni grouping test (α = 0.05).

Gracilaria cornea

		temp (°C)				temp (°C)				temp (°C)	
AA	**day**	**22**	**28**	**AA**	**day**	**22**	**28**	**AA**	**day**	**22**	**28**
Gly (%)	1	5.59	6.17 B,A	Val (%)	1	6.6	9.01 C	Pro (%)	1	3.45	4.45 B
	3	5.47	6.05 B,A		3	6.92	9.34 B,C		3	3.65	4.67 B,A
*day** temp***	7	5.37	5.96 B	*day*** temp****	7	7.74	10.15 B,A	*day*** temp***	7	3.45	4.47 B
	10	5.02	5.61 B		10	6.38	8.79 C		10	3.1	4.12 B
	14	6.37	6.97 A		14	4.08	5.37 A		14	4.15	5.17 A
Thr (%)	1	3.62	4.8 B	Leu (%)	1	6.36	7.93 A	ASP (%)	1	9.95	11.56 A
	3	3.77	4.95 B,A		3	6.49	8.08 A		3	10.38	11.98 A
*day*** temp***	7	4.03	5.21 B,A	*day*** temp***	7	6.36	7.95 A	*day****	7	9.82	11.42 A
	10	3.46	4.64 B		10	5.36	6.95 B		10	7.8	9.40 B
	14	4.47	5.65 A		14	6.91	8.50 A		14	10.72	12.32 A
Ser (%)	1	2.86	4.16 B,A	Ile (%)	1	4.4	6.05 B	Phe (%)	1	3.99	4.96 B,C
	3	2.83	4.10 B		3	4.85	6.49 B,A		3	4.57	5.55 B,A
*day**	7	3.08	4.37 B,A	*day*** temp****	7	5.2	6.84 B,A	*day*** temp***	7	4.12	5.09 B,C
	10	3.46	4.14 B		10	5.36	6.19 B		10	3.8	4.77 C

14	4.08	5.37 A		14	5.59	7.23 A		14	10.72	12.32 A

 Asterix indicates the level of significance '***' 0.001; '**' 0.01; '*' 0.05

As can be seen in **Fig. 5** and **Fig. *6*** the accuracy of prediction of sAA concentration (% of total AA) was reasonable, as indicated by the following statistical parameters: R^2 = 0.962 and RMSE = 0.396.

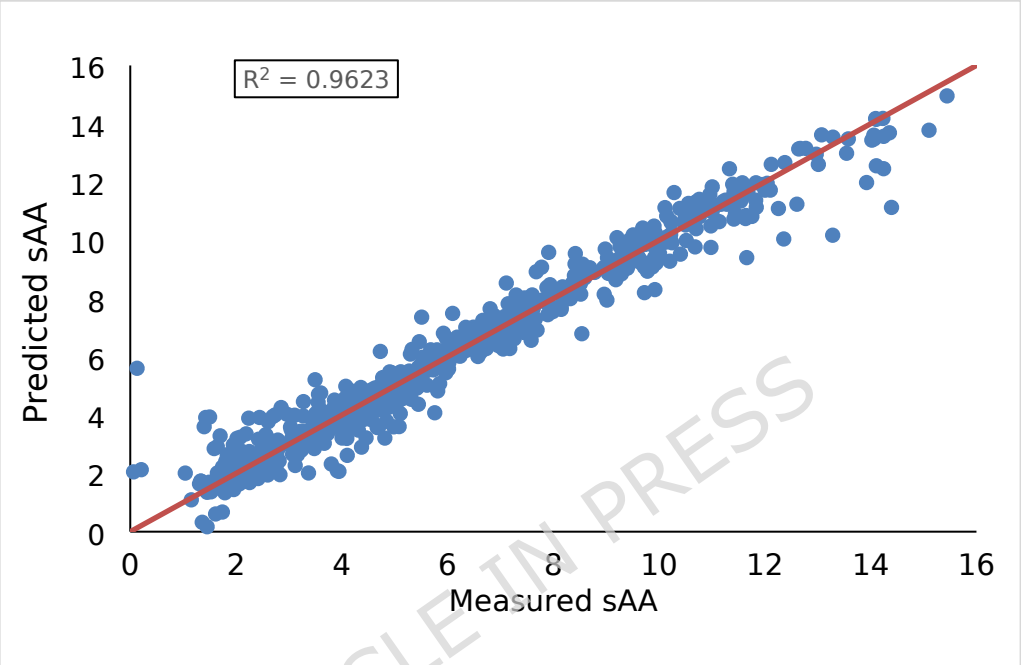


Figure 6. The validation test for the accuracy of the model predicting the proportion of specific amino acid (%).

3.6 Effect of salinity level on phycobiliprotein, thallus phenotype and absorption area

Diffuse reflectance spectroscopy classification of 57 *G. cornea* specimens and features obtained from Kubelka-Munk normalized absorption of the phycobiliproteins (PBS) (including differences in the alga thallus phenotype [17] were identified, and used as an input data for the protein content prediction model. . The model has demonstrated the correlative importance of an absorption in the range 560-670 nm with two prominent peaks around 592-595 nm and 641-643 nm. A distinctive change was obtained in both the *G. cornea* thallus chromophore and in the algae morphology which is demonstrated in **Fig. *7***. It is very clear that under hypersaline conditions on day 10, the turgor pressure is reduced and the red chromophore is much darker in comparison to hypo-and

control conditions and in comparison to the alga phenotype at the starting-point (T0) [8].

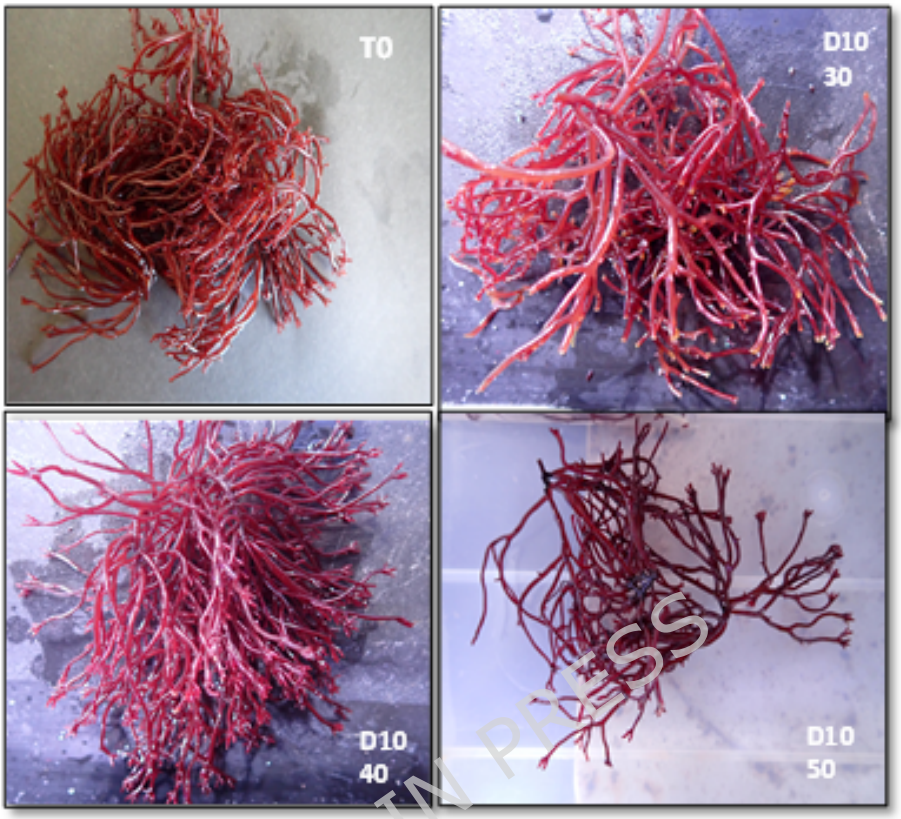


Figure 7. Alteration in thallus morphology and chromophore pigment obtained on day 10 (D10) from *G. cornea* specimens cultured in-door under three salinity levels (30,40, 50 ppt) in comparison to the morphology and pigment of the algae at the starting point (T0).

4. Discussion

4.1 Protein expression

Cultivation conditions, and in particular hypersaline stress and the day of cultivation, clearly contributed to higher protein content in *G. cornea* reaching a distinctive peak on day 14 with more than 35% protein on a dry weight basis (*Bonferroni grouping*, $p = 0.0077$; **Table *1***; ***Fig. 2***). Elevated protein accumulation specifically on day 14, although fluctuated on days before and after, was consistently observed in our previous studies [17, 41], wherein the red algae was subjected to variable cultivation regimes of differential fertilization levels and light intensities across diverse temporal and seasonal conditions. *Gracilaria* rapidly assimilates ammonium (NH_4^+) and nitrate (NO_3^-) during early cultivation. These are converted into glutamine and glutamate via the GS-GOGAT

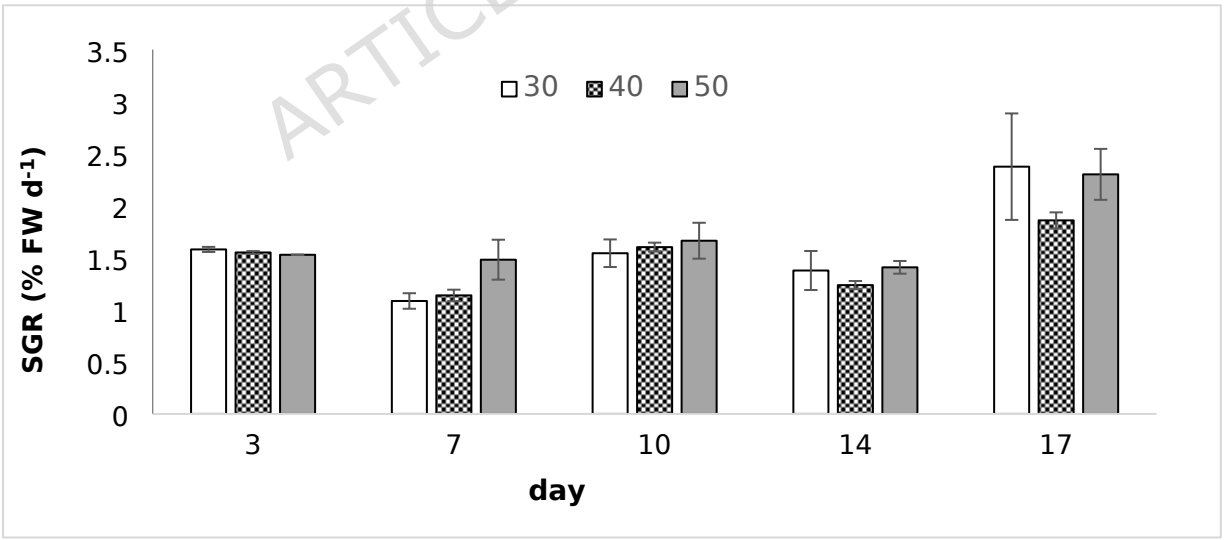
pathway, which are central amino donors for synthesizing other amino acids. This explanation was supported by previous studies [70, 71]. Probably, around day 14, this pathway is highly active, leading to a surge in total protein content. At the same time, the growth rate (***Fig. 8***) has been conventionally regarded as a proxy indicator for productivity, whereas our results show that growth rate, per se, was insignificant in the protein content accumulation response (**Table *2***). This conclusion was valid regardless of salinity level and culture duration. These findings are in line with our previous study in which no correlation could be established between protein biosynthesis and SGR ([17]. Our findings demonstrated that under average ambient temperature of 24.0 ± 0.45 °C and light intensity of 22.37 ± 4.14 µmol photons m^{-2} s^{-1}, the daily growth rate ranged between 1.47% to 1.75% (FW) which is consistent with some previous studies [37] but less than for other species of *Gracilaria,* which demonstrated relatively higher SGR of 4.71% (FW d^{-1}, [72]. Variability in SGR can be attributed to environmental factors such as seasonality, cultivation area, temperature, light intensity and nutrient availability. Hence, the question of why SGR should be such an important consideration in the evaluation of total protein yield, remains valid. The popular equation (Eq. 4) to determine the specific daily growth rate of seaweeds uses the natural logarithm for measuring changes of the ratio of the final fresh weight of the biomass basis to its initial fresh weight. This equation actually simplifies the calculation of growth rates over time. It converts multiplicative biochemical reactions into additive ones, which are generally easier to handle mathematically, but lack the ability to follow nonlinear physiological processes. Another important aspect that must be considered in marine macroalgae is the moisture content, which can account for up to 91% of the biomass in Rhodophyta [3]. It is reasonable to assume that a large part of the overall growth rate derives from moisture accumulation and cell elongation [33]. Higher MC values and the concomitant lower values of the DW:FW ratio actually have a heavy economic implication: for the same yield of the desired product more resources (and hence, costs) are required to transport the biomass and to dry it for further processing [52].

$$\text{SGR (\% day}^{-1}) = 100 \times \frac{\ln(\frac{FW_t}{FW_0})}{t} \quad (4)$$

The results obtained in this study indicate very clearly that *G. cornea* specimens exposed to hypersaline stress and exogenous additions of N and P over time, continued to steadily accumulate protein content concomitantly with growth inhibition. The expression of many proteins during osmotic stress in hypersaline conditions suggests that hypersalinity plays a critical role in regulating algal homeostasis and rapid adaptation [8]. Due to the euryhaline nature of the algae, the acclimation process to salinity stress was apparently fast, with distinctive differences in protein biosynthesis accumulation between salinities, starting from day 1, being manifested in detectable phenotypic changes [35]. According to the model, while hyposaline conditions induced ca. 28% protein content (DW) by day 10, another four days under hypersaline conditions yielded a peak protein content of more than 35%. Therefore day 14 can be considered as the optimal day for harvesting. Despite the distinctive reduction in growth rate, specimens of *G. cornea* cultured under hypersaline conditions, consistently produced a higher DW:FW ratio of 0.30 (in average terms) in comparison to the ratios of 0.26 and 0.27 obtained under 30 and 40 ppt, respectively, thus yielding larger amounts of dried biomass with a higher protein content. Irradiance that was relatively low and ranged between 19 to 24.3 μmol photon m^{-2} s^{-1} induced the same absorption pattern. Spectroscopic peaks appeared in the ranges 590-601 nm and 660-645 nm (***Fig. 9***) within the phycobiliprotein absorbance area regardless of salinity level, suggesting that the phycobiliproteins APC, PC and PE served not only as accessory pigments for light capture, but also as photoprotection chromophores to prevent oxidative stress [37] under hypo- and hypersaline conditions.

Interestingly, protein content in *G. cornea* was maximal under high ambient pH (between 8.0 to 8.1) and high salinities. To a lesser extent this was also observed for hyposalinity (30ppt). In contrast, under natural seawater conditions (40 ppt) protein level was maximal at lower pH (< 8.0). Salinity stress increases photosynthesis activity that is associated with ambient dissolved inorganic carbon (DIC) removal [73] enhancing extrusion of OH^- to the medium explaining the elevated pH level at salinities of 30 and 50 ppt, but not at normal seawater salinity.

Figure 8. Specific growth rate (SGR, % FW) performance of *G. cornea* over time (3-17 days) under different salinity regimes (30, 40, 50 ppt). Bars indicate means ± SD (n = 3).



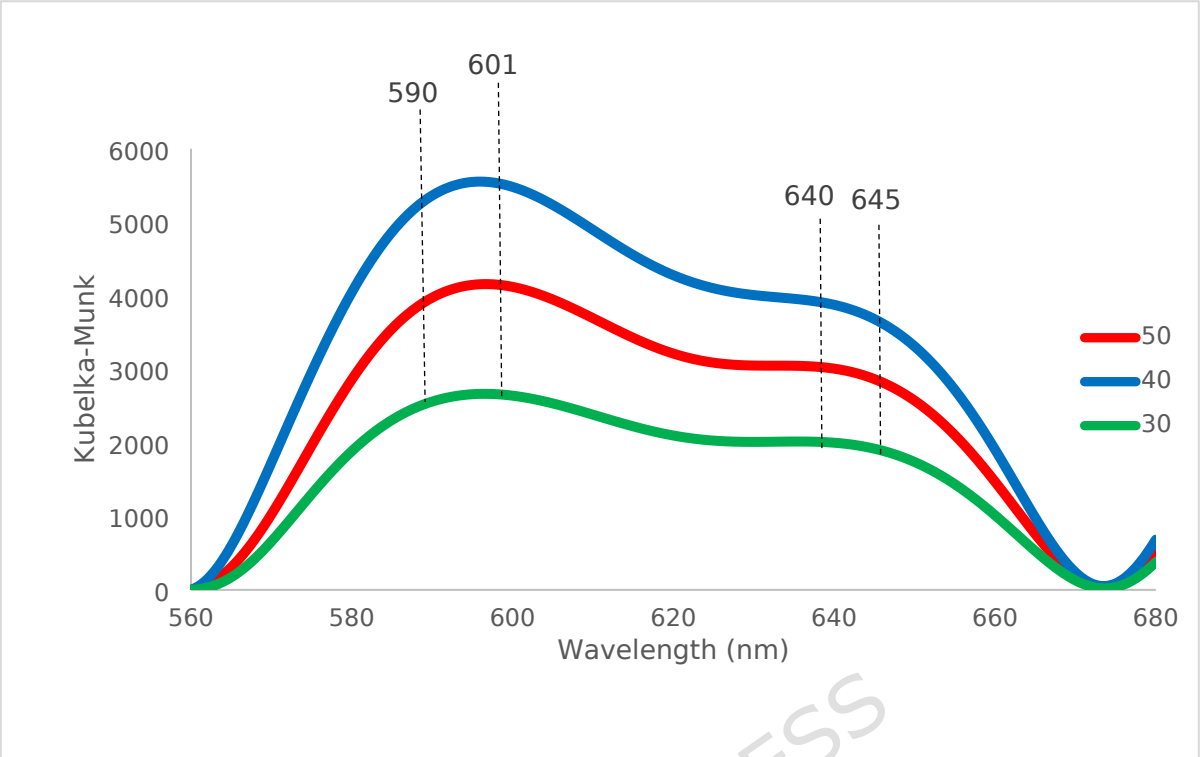


Figure 9. Kubelka-Munk absorbance features [17] of phycobiliproteins in the range 560-680 nm obtained from *G. cornea* thallus measurements cultured under different salinity level (30, 40, 40 ppt). Two distinctive peaks appear at 590-601 nm and at 640-650 nm. Plot represent average data

4.2 Amino acid composition

This study identifies those environmental factors that significantly affect amino acid composition, and for the first time accurately predicts the conditions and specific day of cultivation that foster sAA biosynthesis. This capability is crucial for the evaluation of the nutritional value of algae-derived products for the food industry [12]. The total EAA, which determines the protein quality, was significantly higher ($p < 0.001$) on day 14, exceeding 40% of the total AA, regardless of salinity level and ambient pH, but was significantly affected by the DW:FW ratio ($p = 0.0066$). In contrast, the total NEAA was significantly affected by the day of cultivation ($P < 0.001$), and exceeded 57% on day 17. The EAA/NEAA ratio of 0.84 was highest on day 10. Salinity level was significant for the biosynthesis of tyrosine and serine, which increased under hyper-and-hypo-saline conditions. Some amino acids quantitively increased as ambient temperature rose from 22°C to 28°C reaching their highest level on day 14.

These changes in the biosynthesis of specific amino acids could be an expression of enzyme upregulation or catabolic activity associated with the expression of some proteins, as reported by [74] in his study on the green seaweed *Ulva lactuca.*

Overall, more than 40% of the total amino acid in *G. cornea* consists of EAA regardless of salinity level, which is in line with data for other species of *Gracilaria* [75, 76, 77], higher than other plant-based protein sources such as lupine (38%) and hemp (39.5%) [5], and close to ovalbumin (**Table *4***). For some specific essential amino acid our values even exceeded the recommendations of the major World Food Organizations [78]. However, more research is needed to explore protein digestibility and availability. According to the literature [12, 53, 79], due to rigid cell wall, protein availability is limited and requires processing. Advanced extraction methods and in vitro models, widely discussed in literature, can increase overall digestibility results. For instance, the digestibility rate of protein extracted from *Gracilaria* sp. can reach more than 80%, compared to casein according to [53].

Table 4. Amino acid composition (% of total AA) of G. cornea under different salinity regimes. Values presented are means of triplicates ± SD. nd - not detected

Gracilaria cornea				
Salinity (ppt)	30	40	50	Ovalbumin*
EAA				
Threonine	4.67 ± 0.3	4.13 ± 0.15	4.28 ± 0.4	3.0
Valine	8.73 ± 0.60	7.86 ± 0.29	8.34 ± 0.65	5.4
Leucine	7.39 ± 0.50	6.66 ± 0.29	6.78 ± 0.42	6.2
Isoleucine	5.98 ± 0.43	5.33 ± 0.19	5.53 ± 0.44	4.8
Methionine	2.28 ± 0.19	2.19 ±0.14	2.03 ± 0.24	3.1
Phenylalanine	4.91 ± 0.27	4.53 ± 0.22	4.81 ± 0.46	4.1
Lysine	4.05 ± 0.44	4.18 ± 0.29	4.31 ± 0.41	7.7
Histidine	1.85 ± 0.19	1.86 ± 0.18	1.79 ± 0.15	4.1
Tryptophan	nd	nd	nd	1.0
NEAA				
Alanine	9.49 ± 0.79	8.84 ± 0.27	9.26 ± 0.58	6.7
Glycine	6.68 ± 0.54	6.04 ± 0.18	6.11 ± 0.42	3.4
Serine	4.16 ± 0.77	3.28 ± 0.34	3.82 ± 0.79	6.8
Proline	4.25 ± 0.25	3.81 ± 0.08	3.88 ± 0.35	2.8
Aspartic acid	10.92 ± 0.73	10.10 ± 0.36	10.33 ± 0.86	6.2
Glutamic acid	11.24 ± 0.79	11.61 ± 0.63	12.25 ± 1.09	9.9

Tyrosine	2.04 ± 0.78	3.73 ± 0.71	3.57 ± 0.85	1.8

*[80]

5. Conclusion

As it becomes increasingly evident that humanity must treat the oceans as a sustainable source of protein, researchers must seek ways in which marine organisms can be cultivated to meet the growing demand. Seaweeds are excellent candidates for mariculture as they have little environmental footprint and are nutrient rich. For many years aquaculture played a relatively minor role in global protein production, but its significance has increased over recent years. For mariculture to establish itself with a major global presence, industry must overcome a built-in objective obstacle, namely the fact that the protein density of terrestrial agriculture products is typically much higher than that of marine macroalgae. The challenge facing research is to find and develop improved cultivation systems that can close this gap and bring macroalgae into a more competitive stance as a viable food alternative. Indoor intensive cultivation systems, such as tanks and photobioreactors already employed for agar and agarose production, can be adapted for protein-focused production, with hypersaline conditions inducing natural dewatering that increases protein density per dry weight and reduces post-harvest processing costs. Ecologically, *Gracilaria* contributes to nutrient cycling, carbon sequestration, and sediment stabilization, while its stress-induced metabolites (e.g., osmolytes, phycobiliproteins, antioxidants) provide added commercial value in nutraceuticals and pharmaceuticals. Collectively, these attributes underscore the feasibility of scaling *Gracilaria cornea* cultivation into a sustainable, and climate-resilient protein source for global food security and industrial applications.

In this paper we developed and tested novel tools to manipulate, monitor and assess, both quantitatively and qualitatively, and accurately predict the yield of proteins and amino acid composition in the edible red seaweed *Gracilaria cornea*.

We have also identified the environmental factors that significantly induce the accumulation of these two traits of interest and determined the optimal harvesting day. A high protein yield of more than 35% of the dry weight and natural dewatering responses can be achieved through indoor intensive cultivation under hypersaline conditions, with exogenous addition of nitrogen and phosphorus. We have accurately predicted the proportion of specific amino acid and identified the environmental factors that significantly affect the biosynthesis of both EAA and NEAA**.** These results are crucial for defining protein in terms of nutritional profile and score, and emphasize the feasibility of using marine red macroalgae as a viable alternative protein source for the food industry.

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Data Availability Statement: The spectral and laboratory measurements that support the findings of this study are available from the University of Haifa, but restrictions apply to the availability of these data, which were used under licence for the current study and so are not publicly available. The data are, however, available upon request and with the permission of the University of Haifa.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

NIR	near infrared
ANN	artificial neural network
DW	dry weight
FW	fresh weight
VIS-NIR	visible-near
NM	nano meter
GC-MS	gas chromatography mass spectrometry
AA	amino acid
EAA	essential amino acid
NEAA	nonessential amino acid
ppt	parts per thousand

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