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# Different *Chondrus crispus* Aquaculture Methods and Carrageenan Extraction

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**Abstract:** With the notable scarcity of *Chondrus crispus* on the Portuguese coast, and interest in exploiting compounds such as carrageenan, this study focuses on investigating different aquaculture methods and recording the yield of carrageenan from specimens grown by different methods. We compare the growth of *Chondrus crispus* in aquaculture using Free Floating, Cages, Attempted Fixation on Rock, and Fixed Line similar to Long Line. The best method was Free Floating where Nursery 0 had a 24-day Specific Growth Rate (SGR) of 2.08 ± 0.47%/day. The worst method in terms of growth was Nursery 2 (Attempted Fixation on Rock) where the SGR at 28 days was 0.33 ± 0.69%/day, and no fixation was observed. In terms of carrageenan extraction, all culture methods gave rise to biomass that had a lower extraction yield than wild specimens, at 50.95 ± 4.10%. However, the Free-Floating method from Nursery 1 showed an acceptable carrageenan content (31.43 ± 7.00%). Therefore, we demonstrate that the concept of *C. crispus* cultivation may be key to promoting the sustainability and stability of this species.

**Keywords:** *Chondrus crispus*; SGR; carrageenan; aquaculture; macroalgae



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## 1. Introduction

Seaweed has been part of the human diet for quite some time. Partially consumed seaweed was even discovered years ago in southern Chile which was estimated to be 14,000 years old [1]. Despite this, a large portion of the world's population is still unaware of the benefits of seaweed and holds a variety of misunderstandings about it. Seaweeds are one of the most nutrient-dense food sources on the planet, serving as both food and habitat for a wide variety of marine life forms [2].

Macroalgae are multicellular, macroscopic, eukaryotic, and autotrophic organisms that occupy the maritime habitat and lesser estuarine and freshwater. Being the basis of aquatic food compared to vascular terrestrial plants, they are presented as simple organisms and can be divided into three major taxonomic groups by their characteristics, such as the presence or absence of flagella and the chemical composition of the cell wall. However, the characteristic most used in their grouping and classifying is the presence of specific pigments that clearly identify macroalgae [3]. The presence of different phytopigments is directly related to the different habitats of the species, since not all macroalgae need the same light intensity to perform photosynthesis [4].

In line with this classification, a three-color division has been established: brown macroalgae (Phaeophyceae), red macroalgae (Rhodophyta), and green macroalgae (Chlorophyta) [3].

Marine macroalgae are also considered to be the plant-based food of the future and have even earned "superfood" status—a food market term in recognition of their health benefits, which stem from their superior nutritional profile and abundance of bioactive compounds [5].

The Asian food industry has historically demonstrated strong macroalgae interest, mostly for direct consumption. Meanwhile the same cannot be said for their western counterparts, who mainly exploit macroalgae as a source of functional polysaccharides (carrageenan, agar, and alginates) used as biological additives to give texture and stabilize industrially produced foods, for example [6,7]. The aquaculture of macroalgae is shown to be an efficient method of obtaining the algal biomass needed for a wide variety of products. These products fit into various industries such as food [8], cosmetics [9], pharmaceuticals [10], biofuels [11] and even biopolymers for use in solar panels [12].

The Rhodophyta group (red macroalgae) has the potential, in the future, to be a major source of natural compounds, such as phycocolloids, algal polymers as substitutes for animal gelatin, and even natural pigments for the textile industry [13,14].

The polysaccharides found in the Rhodophyta phylum, agar and carrageenan, are among the most studied and commercially applied algal bio-compounds, and their extraction and purification methods are more advanced in terms of cost-effectiveness and economic viability due to commercial pressure. Agar and carrageenan are used, for example, in the food and pharmaceutical industries as multifunctional additives, such as stabilizers, emulsifiers, and homogenizers, and this has particularly promoted the growth of seaweed aquaculture [15,16].

Within this group of seaweeds exists the species *Chondrus crispus*, also known as "Irish Moss". This red macroalgae grows abundantly on the rocky coasts of the northern and central Atlantic Ocean [17]. It is already widely used for the extraction of carrageenan for food use [18] and exploited for its bioactive properties [8].

The vast majority of *C. crispus* biomass is obtained through harvesting. In Southeast Asia, there is already an effort to produce red macroalgae in aquaculture systems with the objective of insertion into markets that value this species immensely, but there is no such effort in the western market. One of the main sectors of commercialization is the food market with direct consumption of seaweed. *C. crispus* is also an interesting commodity for such a market, being one of the seaweeds with the highest concentration of soluble fiber (15–22%) [19].

The history of *C. crispus* commercialization and interest is well-documented and spans a significant period of time [14,18,20–25].

The utility of Irish moss was well established in Europe at the beginning of the 19th century. Dawson Turner, a British botanist, recorded observations about certain red seaweeds and their composition. He noted that "*Fucus crispus*" was an extremely variable plant in terms of its physical state, as it would fix into a strong gelatinous substance when cooked and cooled down. It would then return to a liquid state when exposed to heat again. Turner also claimed that the algae were already consumed by both Scottish and Irish people [26].

Turner's *F. crispus* is now recognized as *Chondrus crispus*, or Irish moss. This red alga, however, is not a moss and is not exclusive to Ireland. It has been used for centuries by the Irish and other Europeans under a wide variety of common names [27].

The possibility of the further commercial use of Irish moss was recognized in the mid-19th century [28]. Certain aspects of the history of *C. crispus* and its uses have been analyzed [29], and the species has become a model organism for molecular and genetic research in red algae [14].

Interest in Irish moss in North America seems to have grown with the various waves of Irish immigrants to the Boston area in the 18th and early 19th centuries. The need for this algae justified the considerable cost of importing it from Ireland [20,30].

By 1835, there was already abundant growth of the algae on the coast of Massachusetts, and its first American production began on that same coast between 1845 and 1848. A hot-

water extract of the seaweed was typically used as a vegetable gelatin in food preparations such as blancmange, and for its noticeable medicinal and sanitary benefits [31].

Although animal-based gelatin had been known since the end of the Middle Ages, it was mainly used by aristocrats for special occasions, as its production was slow and laborious. The introduction of granulated gelatin by Knox in 1894 drastically affected the price received for dried Irish moss, and commercial harvesting of seaweed became relatively uneconomical in the early decades of the 20th century. Cheap sources of hydrocolloid mucilage from seaweed were readily obtained in Asia at that time [32].

The reduced demand for Irish moss persisted until World War II when Japanese agar became unavailable in 1941. Due to the critical importance of phycocolloids in bacteriology and as food additives, alternative sources were urgently sought both in Europe and North America. The British government initiated a major study on seaweeds that could produce commercial quantities of a mucilage with the required physical and chemical properties [33].

The extract (carrageenan) from Irish moss (*C. crispus*), along with false Irish moss, *Mastocarpus stellatus* (Stackhouse), formerly known as *Gigartina stellata* or *G. mammilosa*, was deemed a satisfactory substitute for agar and consequently marketed as "British agar" [14,18,20,34–39].

Still, due to World War II, seaweed research saw significant developments in Canada, Scotland, and Norway [40].

Japan had entered the war, preventing the supply of phycocolloids from Asia for food and biotechnological uses [32].

Although Irish moss was bleached and dried for exportation from the mid-1920s in Nova Scotia, significant exports of Irish moss from Canada began in 1940 and grew rapidly, increasing the limited supply available from New England [20,37–39,41–43].

Throughout the 1960s and early 1970s, the harvest of Irish moss dramatically increased to meet the growing industrial demand for carrageenan [41]. It was becoming evident that the harvest of Irish moss would be unsustainable in the long run if the demand for carrageenan continued to increase. Likewise, the global supply of carrageenophytes was considered insufficient to support the expanding industry [44]. The global demand for carrageenan and the limited supply of carrageenophytes also interested Professor Dr. Maxwell S. Doty of the University of Hawaii [45], as he stated: "Could the declining population of algae (for hydrocolloid extraction of carrageenan) be increased by marine agriculture or marine agronomy?".

From this moment on, there was a greater interest in the aquaculture of *C. crispus* and other carrageenophytes, but information about its cultivation methods is relatively scarce, since the main details were kept as proprietary information by the companies that developed their growth methods [46].

The focus of this study, therefore, was to achieve sustainable production of *C. crispus* in aquaculture systems. With this in mind, several methods were tested and compared. The quantification of carrageenan in the individuals grown by different methods was also attempted and compared between culture methods and wild individuals.

## 2. Materials and Methods

### *2.1. Macroalgae Collection*

Specimens of *Chondrus crispus* were collected at Praia da Tamargueira, Buarcos, Figueira da Foz (40°10′18.6″ N, 8°53′44.4″ W), Portugal. Samples were collected from areas with well-established *C. crispus* patches and no obvious epiphytes or deterioration. The pools were separated by approximately 1 m, horizontally, to provide the most equal composition and physical state of the seaweed gathered for examination and culture. Once harvested, samples were packed in plastic bags in a cold box and transferred to the culture laboratory where they were cleaned with filtered saltwater to eliminate any sand, pollutants, or other living creatures.

The samples were then separated. Some were weighted and placed in the culture systems, while the remaining biomass was quickly washed with distilled water, dried with absorbent paper, and kept at −18°C for subsequent examination.

### 2.2. Macroalgae Cultivation Techniques

#### 2.2.1. Nursery 0: Free Floating Test

This small-scale trial, which we called a nursery, was conducted in order to gauge the growth of *C. crispus* by the free-floating balloon growth method (Figure 1). For this purpose, biomass was collected on 6 December 2021, at Praia da Tamargueira, Figueira da Foz, Portugal. Specimens were chosen for their small size (blades under 8 cm) and healthy appearance.

**Figure 1.** Nursery 0 in lab, a free-floating method.

Each balloon held 2 L of brackish water and about 4 g of biomass, so as to obtain an approximate ratio of 2 g per L of brackish water. They were placed so as to obtain light between 4000 and 4500 lux, with weak aeration. Both light and aeration systems were connected to a timing plug so that they were on from 6 pm one day to 10 am the next day (16 h light:8 h dark).

Water changes were made weekly, and the new brackish water (from one of the industrial aquaculture tanks) was filtered through a coffee filter so that it contained the least number of impurities and was crystal clear. Weekly weighing was also performed in order to measure biomass growth. To weight the biomass, the algae were removed from the water and patted dry with a paper towel before being placed on the scale. This was performed for all growth methodologies.

Since this was only a test nursery, no water measurements were taken, and therefore the only results obtained were related to biomass weight. This trial lasted for 5 weeks.

#### 2.2.2. Nursery 1: Free Floating Test

For Nursery 1, algae were collected from Tamargueira Beach, Figueira da Foz, Portugal, on 19 January 2022. The individuals were separated by size and distributed so that the smallest individuals (juveniles, blades under 5 cm) were placed in balloons A, B, and C (Nursery 1.1), and balloons D, E, and F (Nursery 1.2) received the largest individuals (adults, blades over 5 cm) (Figure 2).

Approximately 4 g of biomass was placed in each balloon, in order to obtain a ratio of about 2 g of biomass per liter of brackish water. Thus, each balloon held 2 L of brackish water. Starting in this nursery, all the water used in the remaining nurseries was collected from Tordos and filtered through a nylon sock. This choice was made based on the clean appearance of the water and the safety provided by its physicochemical parameters (when measured with a probe). The balloons were placed so as to obtain light between 4000

and 4500 lux, with weak aeration. Both light and aeration systems were connected to a timing plug, so that they were on at 6 p.m. one day and off at 10 a.m. the next day (16 h light:8 h dark).

**Figure 2.** Nursery 1 in lab, a free-floating method.

Throughout this nursery weightings were performed every week (Wednesdays) and water changes were performed 3 times a week (Mondays, Wednesdays, and Fridays). In these water changes, the final ratio was 70% old water to 30% new water. The waters (both new and old) were stored for nutrient analysis (nitrates, nitrites, phosphates, and ammonia) with the help of a photometer (Multiparameter Photometer HI83300, HANNA Instruments, Limena, Italy) and mini checker (Marine Nitrate Checker HI781, HANNA Instruments, Limena, Italy). Every day (possible) measurements of physicochemical parameters (mVpH, pH, mVORP, %DO, ppmDO, mS/cm, mS/cm$^a$, ppt TDS, PSU, σt, °C, and psi) were made with the aid of a multiparametric probe (Multiparameter WP pH/EC/OPDo/ORP HI98494). At the end of the nursery trial with the duration of 4 weeks (16 February 2022), the individuals were removed, a final weighing was performed, and biomass was vacuum packed and frozen for later analysis of carrageenan content.

#### 2.2.3. Nursery 2: Attempt to Attach to Rock Test

For Nursery 2 (Figure 3), specimens of *C. crispus* were collected at Tamargueira Beach on 16 February 2022. These specimens were weighed to obtain about 6 g of biomass for each replicate. Each replicate was tied to a limestone rock weighing between 620 and 750 g, in an effort to attach the algae to it. After this procedure, three replicates (rock and algae) were distributed between two tanks. Thirty-five liters of brackish water (from Tordos, filtered through a nylon sock) were placed in each tank. The tanks were set up for light between 4000 and 4500 lux, with weak aeration. Both light and aeration systems were connected to a timing plug so that they were on from 6 p.m. one day to 10 a.m. the next day (16 h light:8 h dark).

Water exchange was partial (exchange of 10.5 L, 30% of the total amount of water in the tank) on Mondays, Wednesdays, and Fridays. Weighing was performed weekly, every Wednesday. Every day the physical parameters were measured (with the aid of the multiparameter probe Multiparameter WP pH/EC/OPDo/ORP HI98494), and on water change days, the ammonia, phosphates, nitrites, and nitrates levels were measured (with the aid of the Multiparameter Photometer HI83300 and the Marine Nitrate Checker HI781). At the end of the nursery with the duration of 4 weeks (16 March 2022), the individuals were removed, a final weighing was performed, and they were vacuum packed and frozen for later analysis of carrageenan content. Only the values for the initial and final weighing of the nursery are presented, due to the strong variations caused by the weight of the rock. Thus, only the values without the rock present were considered viable.

**Figure 3.** Nursery 2 with an attempt to attach the species to a rock.

#### 2.2.4. Nursery 3 (Nursery 3.1): Reverse Line Nursery Test

For Nursery 3, the biomass of *C. crispus* was collected on 8 March 2022 in Tamargueira Beach. Six specimens were weighed with their attachment organ fixed in their natural extract. These were hung on a Long Line-like system by a string to the surface of the tank water, submerging the alga with its attachment organ and natural extract upwards (Figure 4). The tanks were placed so as to obtain light between 4000 and 4500 lux, with weak aeration. Both light and aeration systems were connected to a timing plug so that they were on from 6 p.m. one day to 10 a.m. the next day (16 h light:8 h dark).

**Figure 4.** Nursery 3 in lab, a reverse nylon cord is crossing the tank to achieve a method close to a long line method.

Partial water changes (30%) were performed every Monday, Wednesday, and Friday, with daily measurement of the physical parameters of the water with the help of a Multiparametric probe (Multiparameter WP pH/EC/OPDo/ORP HI98494). On water exchange days, the old and new waters were analyzed with the aid of a benchtop photometer (Multiparameter Photometer HI83300) and a Marine Nitrate Checker HI781, to quantify ammonia, nitrates, nitrites, and phosphate contents. This nursery had a duration of 4 weeks.

#### 2.2.5. Lines from Nursery 3 at Tordos (Nursery 3.2): Reverse Line Nursery Brought to Outdoor Tank Test

Following Nursery 3, the specimens were transferred to an outdoor line at Tank Tordos for another 4 weeks. This space was chosen because of the constant inflow and outflow of water, similar to the species' natural habitat. Weightings were made every week, and measurements of physicochemical parameters were made with the aid of a probe (Multiparameter WP pH/EC/OPDo/ORP HI98494) whenever possible (Figure 5).

**Figure 5.** Lines From Nursery 3 transferred to Tordos Tank.

#### 2.2.6. Final Fixed Lines

For this method, biomass collection was carried out at Tamargueira beach on 29 April 2022. Selected specimens still had substrate in their attachment organ. They were tied with nylon string and arranged in a similar fashion to the method used for Nursery 3. Weights were taken every week, and measurements of the physicochemical parameters of the water were taken with the aid of a probe (Multiparameter WP pH/EC/OPDo/ORP HI98494) whenever possible (Figure 6). This trial lasted for 4 weeks.

**Figure 6.** Final Fixed Lines underwater in Tordos Tank (such as Nursery 3 at Tordos, but with fresh biomass).

#### 2.2.7. Tank Cages

Using the biomass collected on 19 January 2022 (same as that used in Nursery 1), a system of cages was set up. The biomass was distributed into semi-rigid plastic cages (cages) so that each one weighed approximately 0.5 kg. These were attached to a rope extended (with floats) from one side of the tank to the other. Whenever possible, measurements of the physical parameters of the water were made with the help of the probe (Multiparameter WP pH/EC/OPDo/ORP HI98494) (Figure 7). At the end of the experiment, the biomass was collected and frozen for further analysis. The cage experiment ran for 5 weeks.

**Figure 7.** Cage method in outdoor tank.

### *2.3. Specific Growth Rate and Growth Percentage*

There was a weekly weighing of the algae. The results allowed us to calculate the specific growth rate (a term used to estimate aquaculture production after a certain period) [47,48]:

$$SGR = (\ln(W_f) - \ln(W_i)) \times 100/t$$

where: SGR= Specific growth rate (% weight increase/day); $W_f$ = Weight at final day (g); $W_i$ = Weight at initial day (g); t = time interval between weightings, in days).

This was calculated not only for the optimal SGR (using the initial weight as day 1 and final weight as the last growth showing weightings) but also to calculate weekly SGRs (using a weekly weighting as the initial weight, such as week 3, and the next week as the final weight, week 4).

For an overall Growth Percentage we used the following formula:

$$(Wf - Wi)/Wi \times 100 = Growth\%$$

This indicates the percentual growth of biomass in weight.

### *2.4. Carrageenan Extraction*

Alkaline extraction was carried out using the technique reported by Pereira et al. [49]. The milled seaweed was weighed on a scale (Radwag WLC 1/A2, Radwag, Radom, Poland). Prior to the extraction procedure, the ground seaweed was resuspended and pretreated for 16 h at 4 °C with an acetone:methanol (1:1) solution at a final concentration of 1% (*m*/*v*). Before the extraction procedure, the liquid solution was decanted, and the produced seaweed residues were dried in a forced air oven (Raypa DAF-135, R. Espinar S.L., Barcelona, Spain) at 60 °C.

The samples were immersed in NaOH (1 M) in a hot water bath system (GFL 1003, GFL, Burgwedel, Germany) for 3 h at 85–90 °C. Under vacuum, the solutions were hot filtered through a cloth filter supported by a Buchner funnel and a Kitasato flask. The extracts were then filtered under vacuum using a Goosh 2 silica funnel. Under vacuum, the extract was evaporated to one-third of its initial volume (rotary evaporator model: 2600000, Witeg, Germany). The carrageenan was precipitated by adding twice the amount of 96 percent ethanol to the heated solution. The precipitated carrageenan was washed with ethanol and dried in a ventilated oven for 48 h at 40 °C.

The carrageenan was extracted from 0.7 g of dry biomass, either from samples obtained in Nursery 1 or from the wild. The carrageenan extracted from Nursery 2 and Cages biomass was obtained from 1g of dry biomass. Due to the logistic situation during the study, only these four were analyzed.

To calculate the carrageenan yield percentage, we used the following formula [48]:

Final carrageenan weight (g)/Initial dried biomass (g) × 100 = Carrageenan Yield%

### 2.5. Statistical Analysis

All measurements were performed at least in triplicate, and data were expressed as mean ± standard deviation. All statistical analyses were considered significant at a 5% level ($p < 0.05$). To test for normality and homogeneity of variance, the Shapiro–Wilk test and Levene's F-test were used, respectively. Whenever the requirements were validated (normality and homogeneity of variance), an analysis of variance (ANOVA) and t-tests were performed. If the test values showed significance, a comparison was made, using Tukey's HSD post hoc tests. Whenever the data did not follow normality, non-parametric Kruskal–Wallis tests were performed. Statistical analyses were performed using IBM SPSS statistical software, version 28.0 (IBM Corporation, Armonk, NY, USA).

## 3. Results

### 3.1. Water Physicochemical Parameters

By analyzing the table regarding the physicochemical data (Tables 1 and S2–S8), it is possible to state that there are large variations in the parameters, mainly due to the time of the year when the measurements were taken. In general, the lowest pH was measured in the laboratory, as some days the outside tanks demonstrated a dangerously high pH (and therefore the floodgates were opened to change the tank water).

**Table 1.** Mean physicochemical parameters of water in seaweed cultivation throughout the whole study (±Standard Deviation). For Nursery 1, and Nursery 3, $n = 6$. For Nursery 2, $n = 2$. For Nursery 3 in Tordos, Cage, and Final fixed lines, $n = 1$.

| | mVpH | pH | mVORP | %DO | ppm DO | mS/cm | mS/cm[a] | ppt TDS | PSU | σt | °C | psi |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nursery 1 | −73.05 ± 13.66 | 8.28 ± 0.25 | 132.35 ± 46.69 | 119.88 ±10.65 | 11.31 ± 1.14 | 35.02 ± 1.55 | 26.30 ± 1.42 | 17.51 ± 0.77 | 22.05 ± 1.01 | 16.54 ± 1.00 | 11.94 ± 2.47 | 14.78 ± 0.04 |
| Nursery 2 | −77.59 ± 6.15 | 8.35 ± 0.11 | 146.46 ± 37.53 | 126.97 ± 9.38 | 11.16 ± 0.82 | 39.48 ± 1.55 | 31.18 ± 1.35 | 19.74 ± 0.77 | 25.04 ± 1.49 | 18.66 ± 0.85 | 13.94 ± 0.79 | 14.74 ± 0.10 |
| Nursery 3 | −74.49 ± 6.77 | 8.28 ± 0.12 | 134.13 ± 47.40 | 122.67 ± 3.48 | 10.24 ± 0.38 | 38.99 ± 1.75 | 32.46 ± 1.24 | 19.49 ± 0.88 | 24.90 ± 1.24 | 17.96 ± 1.10 | 16.23 ± 1.10 | 14.68 ± 0.05 |
| Nursery 3 in Tordos | −76.84 ± 4.47 | 8.30 ± 0.08 | 102.05 ± 28.88 | 154.11 ± 17.98 | 11.75 ± 1.11 | 40.03 ± 1.48 | 37.24 ± 1.64 | 20.01 ± 0.74 | 25.61 ± 1.07 | 17.24 ± 1.31 | 21.38 ± 2.58 | 14.78 ± 0.06 |
| Cages | −93.30 ± 20.10 | 8.64 ± 0.38 | 87.14 ± 99.83 | 148.35 ± 27.93 | 13.55 ± 2.55 | 33.57 ± 3.50 | 26.46 ± 2.51 | 16.79 ± 1.75 | 21.12 ± 2.41 | 15.48 ± 2.01 | 13.93 ± 1.75 | 14.88 ± 0.20 |
| Final Fixed Lines | −90.42 ± 7.67 | 8.53 ± 0.13 | 97.94 ± 43.79 | 178.29 ± 34.70 | 12.63 ± 2.24 | 48.05 ± 2.43 | 47.41 ± 2.10 | 24.03 ± 1.21 | 31.33 ± 1.79 | 20.77 ± 1.70 | 24.34 ± 1.83 | 14.89 ± 0.05 |

As for water oxygenation, in the laboratory tanks the goal was to have 120% of Dissolved Oxygen (DO) as per the study of Matos in 2005, but the outdoor tanks always showed a higher DO percentage (about 150) [50].

The total dissolved solids (TDS) were controllable in the laboratory, and since the outdoor tanks were dirt tanks, the TDS value was expected to be higher. The laboratory measurements were always close to 20 ppt TDS, while the Final Fixed Lines method (an outdoor method) presented a mean TDS value of 24.03 ppt TDS. This could have impaired algae growth, stopping photosynthesis due to the solids blocking the passage of light through the water.

In terms of temperature, different values were observed mainly due to the seasonal shifts that occurred during the duration of the study. The Final Fixed Lines Method had the highest average temperature with a value of 24.34 °C. The lowest mean temperature was also in an outdoor tank, in the Cage method, with a value of 13.93 °C. The highest

mean salinity was observed in the Final Fixed Lines method, in which the value presented was 31.33 PSU. The lowest mean salinity (21.12 PSU) was observed in the Cage method. Still, these values were higher than the 19.7 PSU registered by Matos in 2005 [50]. These extremes were expectable, as bigger changes happen in outdoor tanks due to the difficulty in controlling water parameters. These parameters can be influenced by meteorology and tide compatibility in order to change the water in the tanks. The variability in parameters could be a significant problem in this industry for companies that use industrial outdoor tanks.

### 3.2. *C. crispus* Cultivation SGR

When interpreting Table 2, the most consistent weekly Specific Growth Rate (SGR) is Nursery 0, this being the one that would be expected to have the best overall SGR.

**Table 2.** *C. crispus* cultivation SGR in percentage of weight increase per day per week for each method (Mean ± Standard Deviation). For the Cage method, and both variants of Nursery 1 (juvenile and adults: inliers and outliers) $n = 3$, For Nursery 0, Nursery 3 in Tordos, and Final Fixed Line $n = 6$. Different letters indicate significant differences between weeks by each treatment (column) ($p < 0.05$). Nursery 2 was not studied in this table since there are only weight values for initial weight and final weight (weekly weightings were not possible with this methodology).

| Cultivation Type | Week 1 | Week 2 | Week 3 | Week 4 |
|---|---|---|---|---|
| Nursery 0 | $2.036 \pm 0.692^{a.b}$ | $1.511 \pm 1.025^{a}$ | $4.520 \pm 3.426^{a}$ | $0.287 \pm 1.664^{a}$ |
| Nursery 1.1 | $2.266 \pm 0.852^{a.b}$ | $0.593 \pm 0.253^{a}$ | $1.653 \pm 0.621^{a.b}$ | $-0.624 \pm 0.711^{a}$ |
| Nursery 1.2 | $3.290 \pm 0.586^{a}$ | $0.984 \pm 0.334^{a}$ | $1.569 \pm 1.107^{a.b}$ | $-0.728 \pm 0.516^{a}$ |
| Nursery 3.1 | $1.595 \pm 0.424^{a.b}$ | $1.094 \pm 0.084^{a}$ | $-0.256 \pm 0.167^{a.b}$ | $0.583 \pm 0.677^{a}$ |
| Nursery 3.2 | $2.470 \pm 0.922^{a.b}$ | $1.677 \pm 0.966^{a}$ | $-0.801 \pm 1.951^{b}$ | $1.609 \pm 1.030^{a}$ |
| Nursery 3 in Tordos | $0.323 \pm 1.013^{b}$ | $-1.132 \pm 3.912^{a}$ | $1.498 \pm 2.618^{a.b}$ | $1.487 \pm 2.529^{a}$ |
| Cages | $1.011 \pm 0.210^{a.b}$ | $0.395 \pm 0.438^{a}$ | $1.512 \pm 0.962^{a.b}$ | $-1.772 \pm 0.846^{a}$ |
| Final Fixed Line | $2.586 \pm 1.868^{a}$ | $0.872 \pm 1.025^{a}$ | $0.070 \pm 1.355^{a.b}$ | $-0.288 \pm 1.104^{a}$ |

However, there are several negative points throughout the study, which mean that there has been a decrease in biomass. It is also true that for the same methodologies, there are statistically significant differences in some weekly transitions, such as Week 1 to 2 in Nursery 3 in Tordos, and Weeks 2 to 3 and 3 to 4 in the outliers of Nursery 3. These differences within the same growth method were caused by negative values in weekly SGR. This can be explained by the death of parts of the algae or, in the case of tank methods (Nursery 3 in Tordos, Final Fixed Lines, Cages), consumption by fish present in the tanks.

When comparing the SGR values for week 1, only one outcome was statistically significantly different from the rest: Nursery 3 in Tordos. This difference was expectable due to the low value of its SGR for the first week of the trial. This low value can be explained by the transition of already weakened algae (which came from a laboratory tank) to an outdoor tank, while the rest of the methods were conducted with freshly collected biomass.

By studying the maximum value of SGR for each method (Table 1) it was possible to reaffirm that Nursery 0 was the most effective in terms of growth rate. This method was the only one to show statistically significant differences to the five worst maximum values of SGR (Nursery 2, Nursery 3 Inliers, Nursery 3 in Tordos, Final Fixed Lines, and Cages).

According to the results presented of optimal SGR (the best SGR possible to calculate with a minimum of 3 weeks between initial weight and final weight) (Figure 8), the most effective growth method is nursery 0 to 24 days with an SGR of $2.08 \pm 0.47\%$/day. Nursery 1 with adult individuals (replicates D, E, and F) also showed an interesting result with an SGR of $1.94 \pm 0.26\%$/day. These results indicate that the best growth methods are Free Floating, where the algae are agitated through the air pump system. However, these methods were only tested on a smaller scale, and there would be a need for a scale up in the methodology to prove its efficiency.

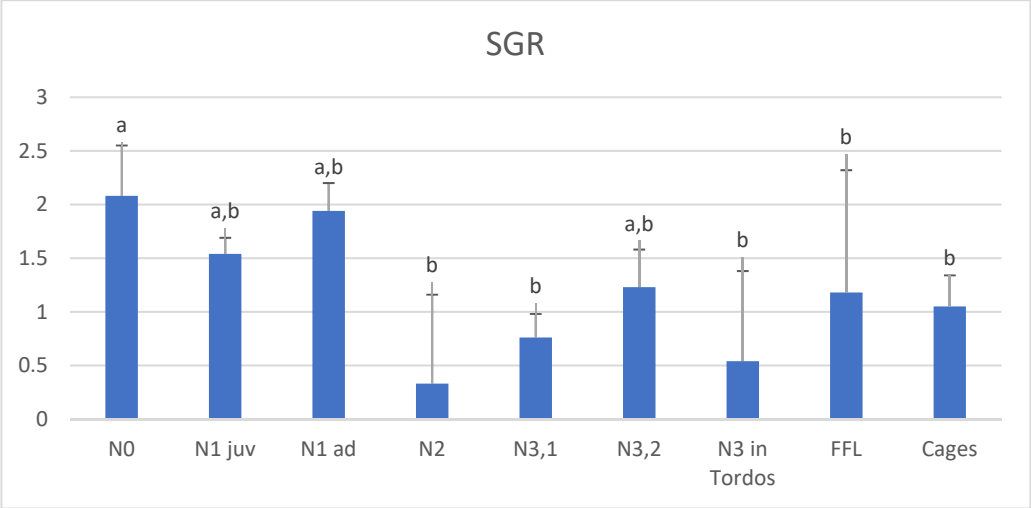


**Figure 8.** Maximum achieved SGR of *C. crispus* cultivation in percentage of growth per day for each method of cultivation tested. Different letters indicate significant differences ($p < 0.05$).

The worst method, according to the overall SGR, was the attempted attachment to the Rock (Nursery 2), which showed an SGR of 0.33 ± 0.69%/day. The attachment attempt itself may have damaged the algae and inhibited the growth of some replicates, explaining the high variability in results.

Another method with a poor SGR was the transition from Nursery 3 to the Tordos Tank (Nursery 3 in Tordos), with an SGR of 0.54 ± 0.84%/day. Since in this method the algae had been in the nursery for a long time (the duration of Nursery 3), one would expect its SGR to be reduced when compared to methods using freshly collected biomass. High variability both in N3 in Tordos and FFL can be explained by possible interactions with the remaining fish in the Tordos Tank, and the contamination of *Ulva intestinalis* in the Tordos Tank (visible in Figure 6).

### *3.3. Global View of Growth*

For easier interpretation of the results, the percentage of growth the algae demonstrated for each method was calculated (Figure 9).

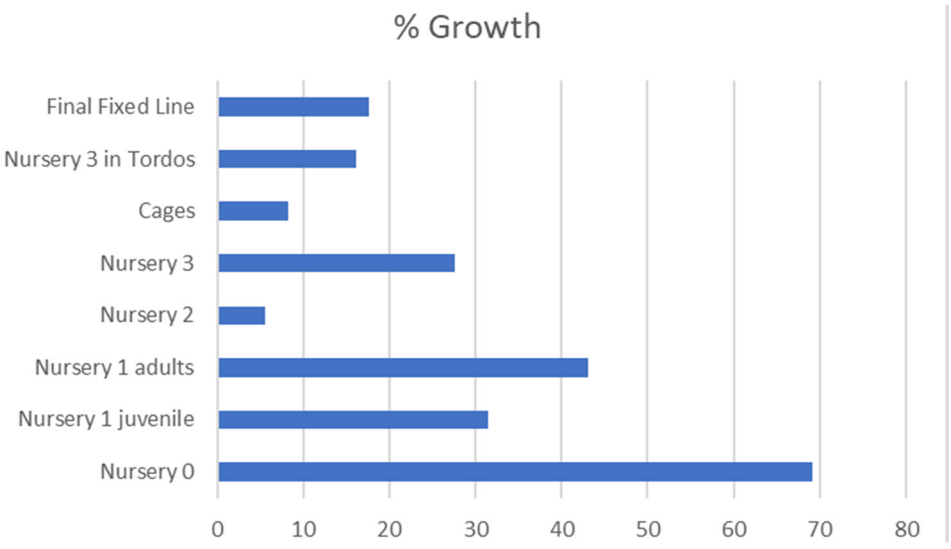


**Figure 9.** Percentage of growth for each method tested at the end of the 4th week.

### *3.4. Carrageenan Extraction*

It was possible to extract carrageenan (Figure 10) in all the methods tested, and even the method with the lowest carrageenan content still has an acceptable percentage (Cage method with a percentage of 25% ± 15.77) (Figure 11). When compared to the wild biomass, a lower carrageenan content is observed in the tested culture methods. This may occur due to the altered properties of the algae in response to being collected from the wild and held in tanks of water with different properties than those found in the wild. Of the extractions made, the highest yields came from the wild (50.95% ± 4.10) and the Nursery 1 method (31.43% ± 7.00). Despite the marked difference, considering that it is increasingly difficult to find *C. crispus* on the Portuguese coast, aquaculture of this species can provide enough carrageenan to sustain the industry without damaging nature, presenting itself as a more sustainable alternative.

**Figure 10.** A thin blade of carrageenan extracted from Nursery 1 Biomass.

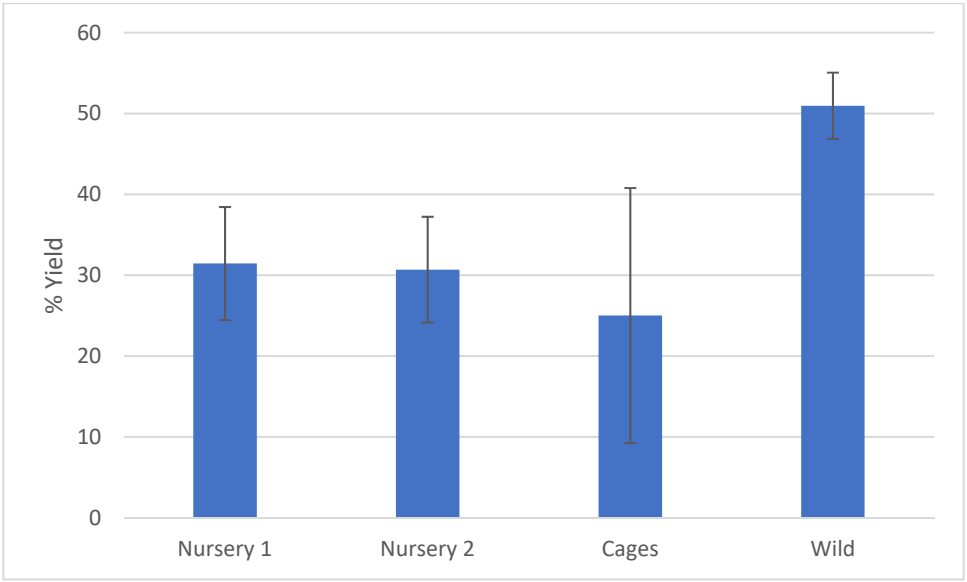


**Figure 11.** Representation of carrageenan yield results in percentage of carrageenan/dry weight for the different methods tested, $p > 0.05$.

### 3.5. Nutrient Analysis

Upon analysis, Tables 3 and S1 allows us to see that, according to the data, there was a strong fluctuation in results regarding nutrient content.

**Table 3.** Mean values of nutrient concentration (Nitrite (µg/L), Nitrate (ppm), Phosphate (µg/L) and Ammonia (mg/L)) per day in the water used thorough Nursery 1, 2, and 3. Water from the frequent water changes was considered, and each measurement was repeated three times.

| | N1 | | | | N2 | | | | N3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Nitrite** | **Nitrate** | **Phosphate** | **Ammonia** | **Nitrite** | **Nitrate** | **Phosphate** | **Ammonia** | **Nitrite** | **Nitrate** | **Phosphate** | **Ammonia** |
| **21 January** | 23.67 | 0.00 | | | | | | | | | | |
| **25 January** | 22.87 | 0.00 | | | | | | | | | | |
| **28 January** | 24.93 | 0.00 | | | | | | | | | | |
| **31 January** | 26.17 | 0.00 | | | | | | | | | | |
| **2 February** | 20.00 | 0.00 | | | | | | | | | | |
| **4 February** | 25.03 | 0.00 | | | | | | | | | | |
| **7 February** | 18.33 | 0.00 | | | | | | | | | | |
| **9 February** | 20.50 | 0.00 | | | | | | | | | | |
| **11 February** | 26.03 | 0.00 | 202.17 | 0.11 | | | | | | | | |
| **14 February** | 23.67 | 0.00 | 983.67 | 0.22 | | | | | | | | |
| **16 February** | 27.03 | 0.00 | 309.33 | 8.25 | 21.67 | 0.00 | 580.00 | 2.42 | | | | |
| **18 February** | | | | | 24.07 | 0.00 | 328.50 | 0.41 | | | | |
| **21 February** | | | | | 27.30 | 0.20 | 612.00 | 9.46 | | | | |
| **23 February** | | | | | 20.23 | 0.00 | 438.00 | 0.19 | | | | |
| **25 February** | | | | | 19.97 | 0.05 | 186.33 | 0.15 | | | | |
| **2 March** | | | | | 22.10 | 0.00 | 435.67 | 4.05 | | | | |
| **4 March** | | | | | 22.93 | 0.19 | 257.67 | 0.22 | | | | |
| **7 March** | | | | | 22.27 | 0.00 | 440.33 | 4.76 | | | | |
| **9 March** | | | | | 25.17 | 0.00 | 219.33 | 5.56 | 28.67 | 0.00 | 233.33 | 5.30 |
| **11 March** | | | | | 10.50 | 0.00 | 550.00 | 0.19 | 28.70 | 0.00 | 288.67 | 0.57 |
| **14 March** | | | | | 18.87 | 0.26 | 586.00 | 0.25 | 15.60 | 0.00 | 343.33 | 0.18 |
| **16 March** | | | | | 20.33 | 0.23 | 655.67 | 3.56 | 22.90 | 0.19 | 273.00 | 3.58 |
| **18 March** | | | | | | | | | 22.57 | 0.07 | 253.67 | 0.10 |
| **21 March** | | | | | | | | | 18.93 | 0.07 | 332.00 | 0.70 |
| **23 March** | | | | | | | | | 25.20 | 0.13 | 323.33 | 5.52 |
| **25 March** | | | | | | | | | 18.83 | 0.26 | 332.67 | 0.25 |
| **28 March** | | | | | | | | | 21.17 | 0.00 | 646.17 | 0.26 |
| **30 March** | | | | | | | | | 28.27 | 0.14 | 477.00 | 3.86 |
| **1 April** | | | | | | | | | 27.77 | 0.00 | 237.67 | 2.83 |
| **4 April** | | | | | | | | | 24.63 | 0.00 | 476.50 | 3.27 |
| **6 April** | | | | | | | | | 25.00 | 0.00 | 286.67 | 3.97 |

The tested method with the biggest reduction in Nitrite was Nursery 3, with a 2.06% average reduction.

As for nitrates, most measurements equaled 0.00 ppm, which indicates that the method used for measuring nitrates should have a higher sensitivity in order to be more accurate in this trial. However, the method with the biggest reduction was, once again, Nursery 3 with a mean reduction of 38.10%. This result was below the expected since previous studies by Corey in 2013 showed a nitrate content reduction of 56.35% in 24 h [51].

Phosphate content was not reduced. In fact, both phosphate and ammonia content increased. The method with the largest content growth was nursery 3—with an average growth of 10.43% in phosphate content—with a mean growth of 240.06% in ammonia content. The nutrient reduction values were not as expected since in previous studies conducted by Kang in 2021, alga of the phylum Rhodophyta (*Gracilariopsis chorda*) were able to reduce phosphate contents by 30.3%, for example [52].

By comparing the SGR results for Nursery 1 (Table 4), with the nutrient reduction results, it is possible to conclude that there was a bigger nutrient reduction in order for the algae to have a bigger growth. In contrast to the results for Nursery 1, when comparing the data of SGR and nutrient reduction for Nursery 3 (Table 5), the lowest value of growth is equivalent to the lowest nutrient reduction, but the opposite (for the highest values)

was not observable. Nonetheless, for the week with the highest growth rate, the nutrient reduction was the second highest in the trial. Therefore, there is a positive correlation between growth and nutrient consumption by algae. This is supported by previous studies such as the study conducted by Abreu in 2011, where the algae studied were *Gracilaria vermiculophylla* (Rhodophyta) in an Integrated Multitrophic Aquaculture system [53].

**Table 4.** Weekly mean SGR in percentage of growth (with standard deviation) compared with the mean percentage of total nutrient reduction (with standard deviation). Values marked with the color green are the highest values, and red are the lowest.

| Week 1 | Week 2 | Week 3 | Week 4 | Week 1 | Week 2 | Week 3 | Week 4 |
|---|---|---|---|---|---|---|---|
| Nursery 1.1 (Juveniles) | | | | Nursery 1.2 (Adults) | | | |
| SGR mean value per week | | | | | | | |
| 2.27 ± 0.85 | 0.59 ± 0.25 | 1.65 ± 0.62 | −0.62 ± 0.71 | 3.29 ± 0.59 | 0.98 ± 0.33 | 1.57 ± 1.11 | −0.73 ± 0.52 |
| Mean Percentage of total nutrient reduction per week | | | | | | | |
| 4.23 ± 4.23 | 2.34 ± 11.60 | −4.65 ± 14.91 | −7.22 ± 13.86 | 4.23 ± 4.23 | 2.34 ± 11.60 | −4.65 ± 14.91 | −7.22 ± 13.86 |

**Table 5.** Weekly mean SGR in percentage of growth (with standard deviation) compared with the mean percentage of total nutrient reduction (with standard deviation). Values marked with the color green are the highest values, and red are the lowest.

| Week 1 | Week 2 | Week 3 | Week 4 | Week 1 | Week 2 | Week 3 | Week 4 |
|---|---|---|---|---|---|---|---|
| Nursery 3.1 (Inliers) | | | | Nursery 3.2 (Outliers) | | | |
| SGR mean value per week | | | | | | | |
| 1.60 ± 0.42 | 1.09 ± 0.08 | −0.26 ± 0.17 | 0.58 ± 0.68 | 2.47 ± 0.92 | 1.68 ± 0.97 | −0.80 ± 1.95 | 1.61 ± 1.03 |
| Mean Percentage of total nutrient reduction per week | | | | | | | |
| 2.22 ± 66.51 | −64.61 ± 218.96 | −152.99 ± 553.9 | 5.05 ± 43.94 | 2.22 ± 66.51 | −64.61 ± 218.96 | −152.99 ± 553.9 | 5.05 ± 43.94 |

## 4. Discussion

Despite the difficulties encountered during the course of these trials, we believe that this work represents the intended methods in a truthful and controlled manner. However, adaptations can be made for future studies.

Many articles consider SGR to be the most standard measure to analyze the potential of cultivation in aquaculture, and Daily SGR allows us to understand how seaweed specimens are developing [54]. Furthermore, it is key to understanding the development of the cultivation. The best example is in Table 2, where the upper limit of the species cultivation is observed (mainly in week 3) before the loss of biomass.

The final growth in % (Section 3.3) demonstrates that controlled conditions boost the seaweed's growth but analyzing week SGR (Table 2) gives an overall insight that it is only different from the other cultivations in week 3, when the most similar Nursery 1 demonstrated different behavior (N1 was rapid growth at week 1, and steady after; N0 was is higher SGR in week 3). This supports that controlled conditions are better for seaweed cultivation, however, it is almost impossible for a company to apply in a large-scale project. However, this technique can be applied to maintain the best specimens to make the large cultivation similar to the Final Fixed Line.

The Nursery 1.2 (adults) had better growth and SGR when compared to their juvenile counterparts, which demonstrates that the theoretical idea that growth is better in juveniles than in adults is not always correct. Field techniques also demonstrate that week 3 is a vital week to harvest the cultured seaweed.

However, as expected, there is a high standard deviation due to environmental factors and the use of an upscaling technique and seawater seaweed in brackish water (thus abiotic factors have a high impact). On the other hand, in the nurseries at the lab, the seaweed demonstrated more stable growth even in brackish water.

After comparing the results with the reported literature, it is possible to conclude that there the tested methods demonstrated a mixed growth rate. A previous study by Ministro in 2020, conducted in 1000 L tanks in Free Floating methodology, obtained an SGR value of

1.31%/day, after the first 14 days of testing [55]. Most of the tested methods surpassed this value in their first week of growth, with the exception of Nursery 3 in Tordos and the Cage method (Table 2). It is not possible to make a direct comparison with Nursery 2, because this method only had weight results at the end of the trial.

The results obtained indicate that, according to the overall SGR, the most effective growth methods are free floating, where the algae are loose and stirred by aeration only. However, these methods have only been tested on a small scale, so another large-scale test would be needed to be sure of the effectiveness and suitability of this method of algae production in an industrial environment [56]. Biomass harvesting is also problematic in this methodology. The data collected from previous experiments with *C. crispus* [55] allowed us to establish a profile and increase rates of growth for this species. This work allowed us to replicate those results and take a step forward in establishing a suitable farming method for *C. crispus* biomass on an industrial scale in a temperate climate. *Chondrus crispus* is a species, as demonstrated by this and previous works, that has slow initial development (SGR = 1.31%), but whose growth rate increases greatly later (SGR = 4.23) in the farming process. As suggested by Zertuche-González in 2001, in order to start the cultivation there is a need for low initial seaweed densities to be exposed to various controlled factors (such as temperature and nutrient availability) [57]. This idea was implemented in the study and resulted in the SGRs achieved. The data indicate that this should be considered when designing a farming strategy and, as such, further development is needed in order to optimize SGR values in large open tanks.

The analysis of carrageenan yield is very important for the study, due to being the most important economic compound in *C. crispus*. There is a possibility that growth in a controlled situation may not be favorable for carrageenan production. Thus, the type of cultivation may impact the polysaccharide yield, as demonstrated by Mendes et al. [58] and Araujo et al. [59], who demonstrated that the seaweed specie can change their polymer production due to the cultivation methods and this is not linear between species (using the same cultivation methods).

As for carrageenan content, there was a notable difference between the optimized aquaculture method tested in carrageenan yield and the wild specimen production. This carrageenan production is in line with the literature, considering that wild seaweed presented higher carrageenan levels than reported in previous works [60–62]. Wild seaweed may have higher carrageenan content because there is an accumulation of carrageenan and overproduction as a response to stress, either by physical stress (being an intertidal species, constantly pushed and pulled by the tide) or chemical (nutrient scarcity). The polysaccharide production may be a survival technique, storing energy in the compound. This is a known survival strategy in algae, which can produce different compounds as a stress response [63,64]. In the aquaculture experiment there was no such stress, with a high nutrient concentration in the water and no tide to induce stress in the algae, therefore there was no increased need for the specimens to produce carrageenan.

This indicates that it is necessary to optimize these methods in order to mitigate the carrageenan decrease in aquacultured algae.

Regarding the reduction in nutrients by the algae, it is possible to interpret that the nutrient concentration influences the growth of the algae in a positive correlation. It might be interesting to repeat this kind of study with a greater focus on nutrient consumption and bioremediation. Our results, however, corroborate with several studies, including the study by Grote in 2016, demonstrating that species of red algae (Rhodophyta) have a high potential for the bioremediation of aquaculture wastewater [65].

From the author's point of view, the most interesting method would be the Final Fixed Line, which showed a relatively good SGR ($1.18 \pm 1.14\%$/day) and seems to be the method most likely to be adapted to a large scale. This is due to the ease of harvesting the algae, the spatial yield per tank, and the apparent maintenance of the algae characteristics. These results seem to be in line with previous experiments where a daily growth rate between 1 and 2% was registered [62].

When reassessing Nursery 2: Attempt to Attach to Rock, it became notable that changes could be made to enhance it and obtain more accurate results if lighter/smaller rocks were used. This would make weighing more precise and facilitate the handling of the algae. The choice of rock can also be an important variable in this attempt.

The variability of water parameters can be a huge problem to overcome in this industry for companies using outdoor tanks. Similar to the problems explored with carrageenan yield rates for the different methods, controlling the multiple factors which impact on seaweed cultivation is a problem to be addressed urgently. The pH, dissolved oxygen, temperature, and dissolved solids are just a few of the factors that need to be under surveillance in a cultivation tank. Maintaining an optimal pH and low water temperatures, dissolved oxygen below 120% and dissolved solids under 20 ppt seem to be the best parameters to maintain and optimize seaweed farming. In light of this, one can suggest that the study would be a better representation of the species in aquaculture if all methods were conducted simultaneously in the same tank/water with similar characteristics and not spaced out. Thus, further development in automated methods to control parameters and research is needed to assess the best methods for each seasonal period [60].

## 5. Conclusions

This study corroborates other studies which state that various environmental and chemical factors influence the growth of macroalgae, whether in the laboratory or outdoors.

We believe this study takes us one step closer to the definitive industrial-scale aquaculture method for this species. Establishing such a method would be important for the sector primarily because it allows for increased production variability for companies, increasing their competitiveness [57]. By introducing an edible species (such as *C. crispus*) with a rich biochemical and nutritional profile into seaweed aquaculture located in more temperate climates, we will allow these companies to not only increase available products but also to take advantage of this and create new market opportunities and/or new products. As already mentioned, *Chondrus crispus* is a seaweed rich in proteins, vitamin A, PUFAs, mineral salts and phycocolloids, namely carrageenans, which are all products suitable for markets such as human nutrition, cosmetics, and pharmaceuticals, amongst others. One of the problems which requires attention is that this work detected lower production of carrageenan in the cultivated biomass as opposed to wild specimens. It was then hypothesized, after analyzing the data collected from the various cultivation methods, that it could occur following a loss of viability due to the maintenance of seaweed in tanks with water that has different physicochemical characteristics from the collection site.

From a future perspective, aquaculture and general study of this species are necessary for its protection and to obtain bio-compounds of interest, therefore increasing its value in the market and making its aquaculture even more desirable. This study can serve as a base point for future studies in the field, and the methods tested in this study can be reused and adapted for other similar species, adding to our known methods of algae aquaculture.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app13095466/s1, Table S1. Nutrient values for the new water used when changing water, N1, N2 and N3, Table S2. Multiparametric Probe values for the new water introduced in the nurseries, Table S3. Multiparametric Probe average values for the water in N1 (before changing waters), Table S4. Multiparametric Probe average values for the water in N2 (before changing waters), Table S5. Multiparametric Probe average values for the water in N3 (before changing waters), Table S6. Multiparametric Probe average values for the water in Tordos during N3 in Tordos, Table S7. Multiparametric Probe average values for the water in Tordos during Final Fixed Lines, Table S8. Multiparametric Probe average values for the water in Tordos during cages method.

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

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