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Influence of pH, N, P, N: P Ratio, and Dissolved Inorganic Carbon on *Ulva ohnoi* Growth and Biomass Quality: Potential Implications in IMTA-RAS

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Abstract: *Ulva ohnoi* has a big potential in IMTA-RAS fish–seaweed systems. In order to design the best production strategy in these systems, the effect of the main environmental factors, such as pH, nutrient concentration (N, P, and N: P ratios) and dissolved inorganic carbon (DIC), on the productivity, bio filtration capacity, and quality of the biomass obtained was studied. It is concluded that in closed systems, strong pH variations (7.9–10.1) do not influence the growth of *U. ohnoi* and growth is slowed down due to the depletion of DIC. This fact would not be a problem in IMTA-RAS fish–macroalgae systems, due to the physiological activity of the fish contributing CO_2 to the medium and replenishing it. The results obtained in the wide range of N: P ratios tested (2–410), allow us to conclude that this ratio should not be a limiting factor for the cultivation of *Ulva ohnoi* in IMTA-RAS systems. Based on those results, the best strategy to follow in an IMTA-RAS sole–sea lettuce would be to maintain the algae with highest level of nitrogen. This procedure implies a high rate of water renewal, which would also guarantee the maintenance of an adequate DIC and the best commercial quality of seaweed.

Keywords: *Ulva ohnoi*; IMTA-RAS; pH; DIC; DIN; DIP; N: P ratio

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1. Introduction

Green seaweeds belonging to genus *Ulva* Linnaeus (Ulvophyceae, Chlorophyta) have been identified as good candidates for filtering fish effluents due to their capacity to be cultured unattached, their wide environmental tolerances [1–3], together with their high growth rates and high removal rate, and removal efficiency to bio filtrate N and P of the water [4,5]. Furthermore, *Ulva* is gaining interest as an ingredient for animal feed, mainly in aquaculture [6,7]; as a source of ulvans, sulphated polysaccharides with biomedical uses in tissue engineering and regenerative medicine [8]; and also, for its antiviral, antioxidant, anticoagulant, antihyperlipidemic and anticancer activities, in addition to immunostimulatory effects in the formulation of functional foods [9–12].

After the recent works [13,14] that cleared up many of the identification issues of the *Ulva* species from the European coast, the most frequent laminar species in the temperate Atlantic and Mediterranean coasts of the Iberian Peninsula are: *Ulva lacinulata* (Kützing) Wittrock (which includes the records of *U. laetevirens* Areschoug and many of *U. rigida* C. Agardh), *U. lactuca* Linnaeus (including those of *U. fasciata* Delile and *U. rotundata* Bliding), and *U. ohnoi* M. Hiraoka & S. Shimada. There is increasing evidence that *Ulva* species are typically cryptogenic, making it virtually impossible to know their true origin. If we compare the descriptions of *U. rotundata* [15] and those of *U. ohnoi* [16], they seem to belong

to the same taxon. Therefore, it is very likely that *U. ohnoi* is an autochthonous species or already introduced in the past on the South Atlantic and Mediterranean coasts of the Iberian Peninsula. In fact, *U. rotundata* was always cited on this coast until, in 2002, after the morphological and molecular description of *U. ohnoi*, this last taxon, described from the Pacific, was cited as one of the most frequent species in marshland environments in the South of the Iberian Peninsula [17]. *Ulva ohnoi* has been suggested as one of the most suitable species for land-based cultivation, due to its high growth rate in temperate waters, biomass productivity, nitrogen and phosphorous bio filtration capacity, and commercial quality [10,18–22].

Solea senegalensis Kaup, 1858, has shown to be an adequate fish species for growing at high densities, compatible with those needed for its intensive commercial farming [23]. The rising intensive production of this species in Spain, Portugal and France [24] has increased the interest on developing specific land-based integrated multi-trophic aquaculture (IMTA) technologies to minimize the environmental impact. The similar range of optimal temperatures for *U. ohnoi* and *S. senegalensis* cultures generates great expectations on the feasibility of integrating the production of both species. One of the main challenges to expand the introduction of IMTA technologies is related to the capacity to increase the algae productivity and nutrient uptake per surface unit, reducing the land area required. Fish–algae IMTA recirculating aquaculture systems (IMTA-RAS) allow the intensification of fish production and provide high amounts of nutrients whose concentration in water can be controlled with the water flow rate delivered from fish to algae tanks. In order to be able to design the best and most profitable strategy for the management of these systems, it is necessary to examine the influence of the different environmental parameters that could modify the productivity of *U. ohnoi*, its bio filtration capacity and the commercial quality of the harvest obtained.

It is known that temperature and light intensity are the physical fundamental factors determining seaweed productivity. The first of them is imposed by the conditions of cultivation of sole (18–22 °C) and is not modifiable in a profitable way. The second, light intensity and dose, is highly dependent on the density of culture, design of the tanks and the movement of algae in them [22].

Chemical factors such as pH, DIC and levels of N, P, and N: P ratio of the water can become fundamental parameters, especially in a recirculating aquaculture system (RAS), where renewal tends to be limited. Different pH levels play a very important role in photosynthesis and carbon sequestration in terrestrial plants and in macroalgae.

Seawater in equilibrium with the atmosphere at about pH 8.2, contains about 2.2 mM of dissolved inorganic carbon (DIC). The most important carbon species is HCO_3^-; CO_2^{3-} is less than 200 μM, and only 10 μM is in the form of CO_2 [25]. Most seaweeds tend to use bicarbonate ion (HCO_3^-) as a source of carbon in water, through different metabolic processes, mainly by either extracellular, carbonic anhydrase (CA)-mediated, dehydration to form CO_2 or by direct uptake/transport via an anion exchanger (AE). These two ways of bicarbonate utilization are not mutually exclusive, but both may be present in a given *Ulva* species. Inside the cells, all inorganic carbon must ultimately be converted to CO_2 for fixation through the enzymatic action of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the main enzyme for carbon fixation [26–28]. In confined environments, the assimilation of carbon both in the form of CO_2 or HCO_3^- during the photosynthetic process raises the pH of the medium by altering the balance of CO_2, HCO_3^- and CO_2^{3-} and can deplete the alkaline reserve of water. Inorganic carbon is a limiting growth factor [29] and low availability of CO_2 and HCO_3^- can drive harmful impacts on algae metabolism (e.g., photosynthesis, respiration and nutrient uptake) and thus, their productivity [1,30,31]. No previous data are available on the influence of the gradual depletion of DIC due to the growth of *Ulva* itself in closed recirculating systems. In natural environments this phenomenon does not usually happens because during regular tide cycles a total water renewal occurs.

Regarding the pH, in general, open-sea species are very stenotic to variations, as pH remains very constant in seawater, due to the buffer effect of the carbon balance previously explained. However, species of saltmarshes or those that tend to live in confined environments during low tide, as many species of the genus *Ulva* very frequent in tide-pools, can tolerate the wide variations in pH typical of these environments [28], without apparently affecting their viability, although having an effect in productivity [32].

As for nutrients, the concentrations of N and P in RAS systems are much higher from those of natural environments. In RAS systems, the main parameter that is usually controlled in the water is the concentration of ammonium due to its high toxicity for fish [33]. Seaweeds are able to assimilate N in several forms (ammonium, nitrites, nitrates). In different species of seaweeds high levels of N in the medium have shown a positive relationship with protein content [34–36]. On the other hand, P levels, although high in RAS (several ppm; 2–3 orders of magnitude with respect to the natural environment), are not considered toxic to fish; however, this element and the N: P ratio do have a great relevance in the seaweed development [27,28]. Systematic differences in P requirements, uptake kinetics and P storage capacity occur when comparing fast-growing and slow-growing seaweeds. Species with fast growth, as *Ulva* spp., cannot rely on stored P for long, and therefore their performance requires a continuous availability and supply of dissolved inorganic phosphorous (DIP), whereas algae of intermediate and slow growth rely more on stored P during periods of low DIP availability [37].

In the past decades the potential for the commercial cultivation of *Ulva* spp. has been evaluated in different semi-enclosed culture systems, although most of these works were only focused on general biological descriptors and culture physic-chemical variables [30,31,38,39]. Different physiological aspects of cultivated macroalgae have been assessed previously [23,24]. Moreover, changes in the growth rate and chemical composition in *U. ohnoi* were examined under different light, temperature and nitrogen conditions [5,40], but the available information for the cultures of this and other seaweed species is still limited [11,39].

In order to establish guidelines for the optimization of the culture of *U. ohnoi*, the main objective of this work was to carry out a study of the influence of the variations in pH, DIC, levels of N and P, and their ratios on the development and productivity of *U. ohnoi*. Knowing the influence of those parameters is very relevant, as can vary significantly depending on the type of the IMTA systems (IMTA-RAS, open or semi-enclosed) and tend to be very different from those of the natural environment.

2. Materials and Methods

2.1. Ulva ohnoi Clone: Morphological, Compositional and Physiological Characteristics

Ulva ohnoi was collected at the Parque Natural de las Marismas del Odiel, Huelva, Spain (latitude, 37°14′ N; longitude, 6°59′ W). This species was genetically identified by DNA extraction and PCR amplification of the chloroplast rbcl gene following the protocol described in Hayden et al. [41] with the primers used by Manhart [42]. It was maintained at the Laboratory of Applied Phycology of Advanced Scientific Research Center (CICA) of the University of A Coruña (Spain), for more than four years, in "tumble culture" with aeration from the bottom of the tanks. To ensure the active growth of the clone in the stock tanks, a weekly change of culture medium was made and the density was adjusted to 3 $kg \cdot m^{-3}$. Fresh weight was obtained after excess water had previously been removed by spinning in a salad spinner for 3 min before weighing.

In order to evaluate the possible variations in the cellular morphology of *U. ohnoi* in the different experimental conditions (cell size and disposition of the plastid, number of pyrenoids, etc.), observations were made in superficial view of the lamina of selected specimens with an optical microscope (Olympus BX50 associated with a photographic camera).

The elemental analysis was performed by a certified laboratory of University of A Coruña (SAI-UTIA—UDC Research Support Services) and according to the procedures described below. Three blade samples were randomly collected from different treatments at

the end of experiments for determination of carbon, hydrogen, nitrogen and sulfur (C, H, N, S, respectively) content using an elemental analyzer FlashEA 1112 (Thermo Finnigan, Milan, Italy) and according to the standard procedures in use in this unit. The following factor was used to determine the protein content conversion: Proteins (%) = % N × 6.25 [40].

In the different experiments *Ulva ohnoi* growth was calculated according to the following specific growth rate equation (SGR) [43]:

$$\text{SGR (\%FW·day}^{-1}) = 100 \times 1\ [(\text{LnWt} - \text{LnWo})/\text{t}]$$

where Wo and Wt are the initial and final weights and t is the number of culture days.

The DIN (dissolved inorganic nitrogen) and DIP (dissolved inorganic phosphorous) bio filtration capacity of *U. ohnoi* was presented in two ways: Removal Rate (RR, mg L^{-1}·t^{-1}), and the Removal Efficiency (RE, %) by means of the following equations:

$$\text{RR} = (\text{Co} - \text{Ct})/\text{t};\ \text{RE} = [(\text{Co} - \text{Ct})/\text{Co}] \times 100$$

where Co and Ct are the initial and final concentrations of DIN or DIP, and t is the number of culture days.

2.2. Measurement of Basic Water Parameters

The DIC of the water was calculated by measuring the total inorganic carbon in the water sample after shifting the carbonate-bicarbonate-carbon dioxide equilibrium towards carbon dioxide by lowering the pH to 4.5 with the addition of citric acid. CO_2 was measured using a CO_2 probe (OxyGuard®).

To determine the concentrations of DIN and DIP, a UV-visible spectrophotometer (Agilent 8453, Santa Clara, CA, USA) was used. The determination of DIN was performed according to method 4500-NO_3.B, described in [44] and the samples were 1:10 diluted (culture media: distilled water) since the method is only linear until concentrations ≈10 mg N L^{-1}. The analytical quality control was ensured by using calibration curves (0.2, 0.4, 0.8, 1, 2, 4, 5, 7 mg N L^{-1}), that result from running standard solutions at the beginning and in parallel with blanks and samples. Determination of DIP was performed following the method described in [45], a colorimetric method based on reduction of phosphomolybdic acid for dissolved inorganic phosphate, with the analytical quality control being ensured as described above for determination of DIN (calibration curves—0.1, 0.2, 0.5, 1 mg P L^{-1}).

2.3. Influence of the Variations in pH and DIC

The evaluation of the influence of pH variations and DIC on the culture of *U. ohnoi* was carried out using a suspension tank culture system ("tumble culture"). For this, two tanks of 100 L were used, with a volume of 50 L of *Ulva* culture medium each tank and a constant aeration system. Lighting was provided with Prilux ecSaver High Luminosity lamps of 85 W and 6400 °K with 300 µmol m^{-2} s^{-1} of light on the surface of the tank, a photoperiod of 12:12 h light: dark. Salinity was established at 34‰, compensating evaporation losses by adding freshwater. Continuous measurement of pH and temperature were obtained from a probe in each tank (HANNA Instruments) and recorded every 15 min by means of a data-logger. The water temperature was kept constant at 18 ± 1.5 °C using submersible heating systems (EHEIM thermocontrol, 150 watt). One of the two tanks used in the experiments, the control tank, also had an automatic DIC regulation system associated with the pH measurement by injecting CO_2 through a diffuser located at the bottom of the tank. *Ulva* culture medium consisted of raw seawater filtered at 40 µm enriched with NO_3Na and $PO_4H_2Na{\cdot}2H_2O$ to a final concentration of 20 mg L^{-1} N and 0.5 mg L^{-1} P and oligo elements (Fe, Mn, Zn and Co). These nutrient concentrations mimic the ones on the effluents of a sole culture.

One experimental condition was "controlled pH" (C) (max. 8.4) by diffusion of CO_2 and the other "uncontrolled pH" (UC). Three consecutive experiments of one week duration were carried out, each week corresponding to an experimental replica (n = 3). In order to

achieve marked variations in pH and DIC between conditions, experiments were started with a relatively high density (4 kg m^{-3}), corresponding to 200 g of fresh weight (FW) of initial biomass of *U. ohnoi* from the stock in the 50 L.

FW was measured daily. DIC, DIN and DIP were measured at the beginning and the end of the experiment (one week). *U. ohnoi* cellular morphology was observed at the end of the experiment and the mean C and N content of *Ulva* biomass was measured.

2.4. Influence of N, P Levels, and N: P Ratio

The effect of N and P concentration on *U. ohnoi*, as well as the possible interaction between both nutrients, was studied in small-scale cultures in multi-well plates (Falcon®) with six wells. A 2 cm diameter *U. ohnoi* disc, obtained with a punch from the blades of the thalli from the stock culture, was placed in each well with 10 mL of culture medium. The plates were kept in a controlled environment chamber (Liebherr) at a temperature of 18 ± 0.2 °C, illumination of 300 ± 20 µmol m^{-2} s^{-1}, using Samsung VT-1422 LED panels (6400 °K), light: dark photoperiod of 12:12 and orbital shaking at 50 rpm using a Skyline Shaker Dos-20L.

Prior to the experiment, an approximately double number of the discs to be used were kept acclimatizing for 72 h in the same chamber under the same nutrient conditions as the stock and without medium renewal. After acclimatization, the discs were weighed fresh (after drying by contact with filter paper) and selected based on the mean weight to reduce the possible variability in initial wet weight. These discs were finally randomly placed in the wells of the different plates.

In order to study the independent effect of N and P and the possible interactions, an orthogonal bifactorial 3 k design was used. The combination matrix of these two factors (N, P) was built at three levels: 2, 42 and 82 ppm of N and 0.2, 0.6 and 1 ppm of P (n = 1), with four replicates in the average conditions of 42N/0.6P (n = 4) for the statistical analysis, assuming their variance as the experimental variance for all the levels (Figure 1).

<table>
<tr><td>2/0.2</td><td colspan="2">2/0.6</td><td>2/1</td></tr>
<tr><td rowspan="2">42/0.2</td><td>42/0.6</td><td>42/0.6</td><td rowspan="2">42/1</td></tr>
<tr><td>42/0.6</td><td>42/0.6</td></tr>
<tr><td>82/0.2</td><td colspan="2">82/0.6</td><td>82/1</td></tr>
</table>

Figure 1. Matrix of the levels of N and P used in each experiment. Note that of the average conditions (42 ppm N and 0.6 ppm P) four replicates are established.

Four identical experiments were carried out (two + two due to the physical impossibility of performing all four at the same time), each lasting 28 days. In each experiment, 24 discs were used (two replicas of each level and eight of the average conditions). In all experiments, the nine different culture media were renewed daily. The first two weeks were considered acclimatization to the different culture conditions, so at the end of them the discs were trimmed and weighed again to keep them in culture for another two weeks, considered as those of the experiment.

The studies of assimilation rate of N and P, morphology, percent of dry matter and elemental composition were carried out only at the 2/0.2; 46/0.6 and 82/1 ppm levels of N and P, respectively, and in the last two experiments. For the estimates of the RR and RE of N and P in the changes of medium, samples of each of the levels referred to three, six, nine and twelve days of the last two weeks the cultures were kept. At the end of the experiment, the discs of these levels, after obtaining their wet weight, were washed with distilled water, dried and placed in an oven at 45 °C until constant weight in order to calculate the percent of dry matter and proceed to its elemental analysis of C, H, N and S.

2.5. Statistical Analysis

For all the parameters analyzed, the means and their standard error were calculated. The statistical treatment of the data was carried out using the Minitab 18.1 program, and the graphs were made with the Microsoft© Excel program for Windows and Minitab 18.1.

The initial hypothesis that pH, growth rate, weight gain, DIC and nutritional composition are not different between the different experimental conditions, was analyzed by one-way analysis of variance (ANOVA) at a confidence level of 95% (p-value < 0.05).

3. Results

3.1. Influence of the Variations in pH and Alkaline Reserve

As can be seen in Figure 2, significant differences in the pH values in the controlled and uncontrolled pH condition were observed; the mean pH value for both conditions was 8.16 ± 0.3 and 9.45 ± 0.5, respectively. In the controlled condition (C), the daily variations in pH were a maximum of four-tenths (7.9–8.3), while in the uncontrolled condition (UC) they were much larger (7.9–10.1). A progressive increase in the average pH of the culture in the light phase and a smaller decrease in the dark phase were observed in UC in the three experiments.

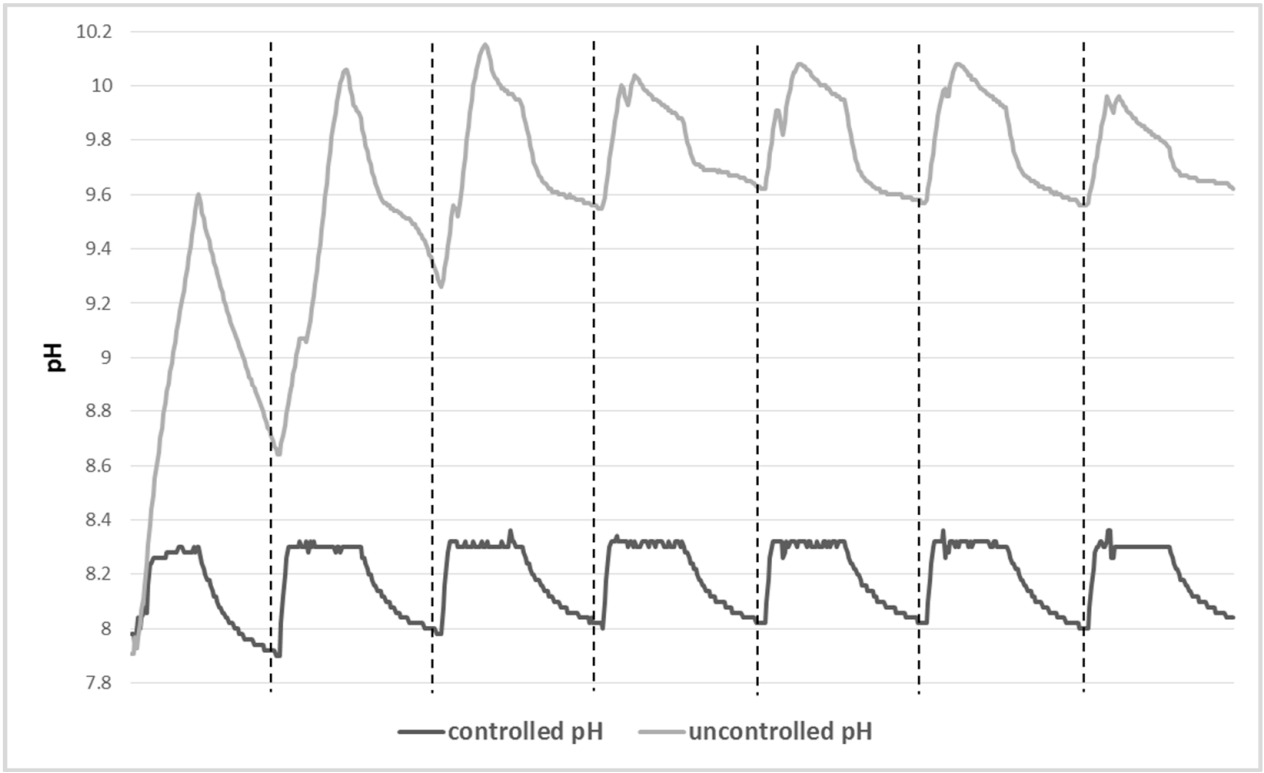

Figure 2. Average evolution of pH during the seven days of the three experimental weeks in both conditions.

Regarding the increase in biomass (FW) in both conditions (Figure 3), significant differences between the two conditions were observed that at the end of the experiment, with greater FW in the C than in UC. However, these differences were only statistically significant from the fourth day of culture.

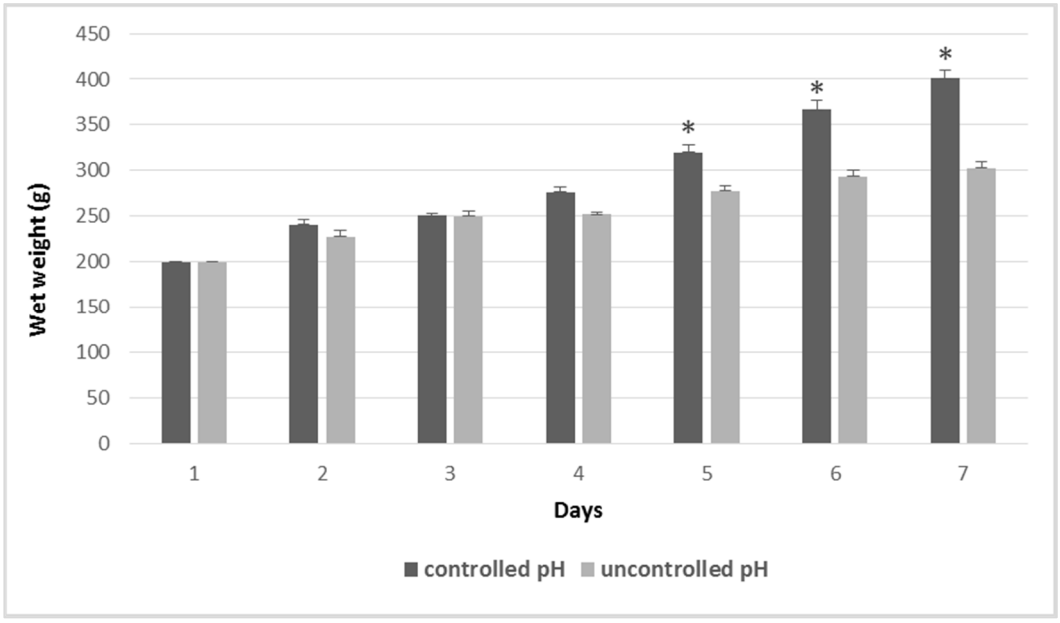

Figure 3. Wet weight (g) and standard error in the different experimental conditions during the culture period. Statistically significant differences ($p < 0.05$), showed by asterisks (*) were observed from the 4th day.

Daily growth rates also showed statistically significant differences between both conditions (Figure 4), but in this case the trend of these differences throughout the cultivation period is much more erratic, especially in the first three days, as significant statistical variations between the conditions were found only on the 3rd and 5th days of culture.

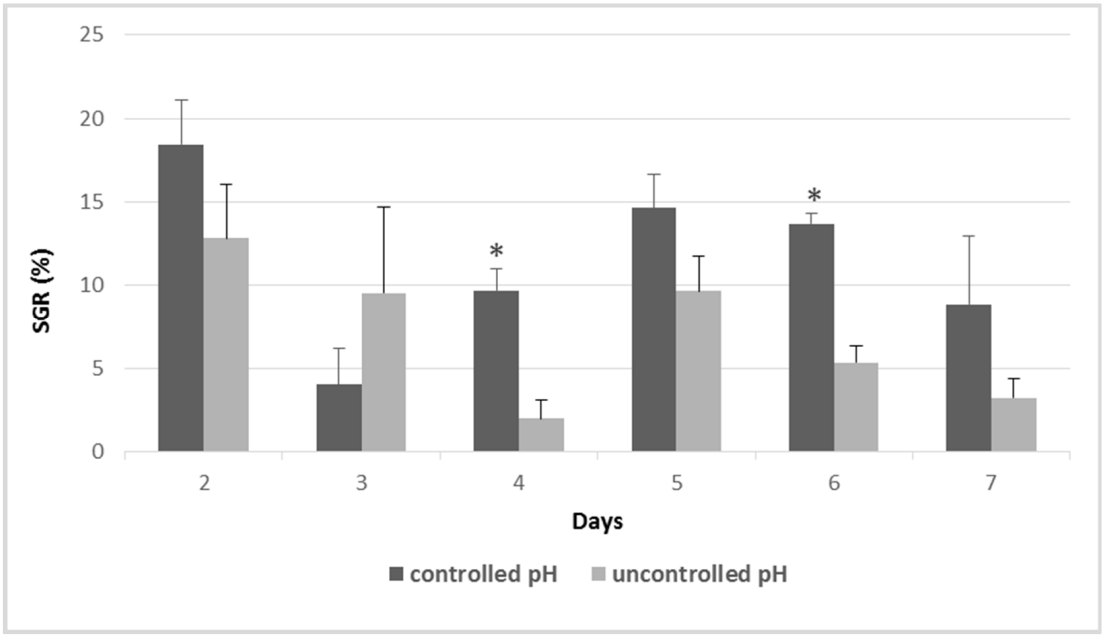

Figure 4. SGR and standard error in the different experimental conditions during the culture period. Statistically significant differences ($p < 0.05$) are showed by asterisks (*).

In Figure 5 it can be seen that in C condition the optimal concentration of DIC was maintained (over 100 mg L^{-1} CO_2), while in the UC condition DIC was significantly reduced at the end of the experiments (over 40 mg L^{-1} CO_2).

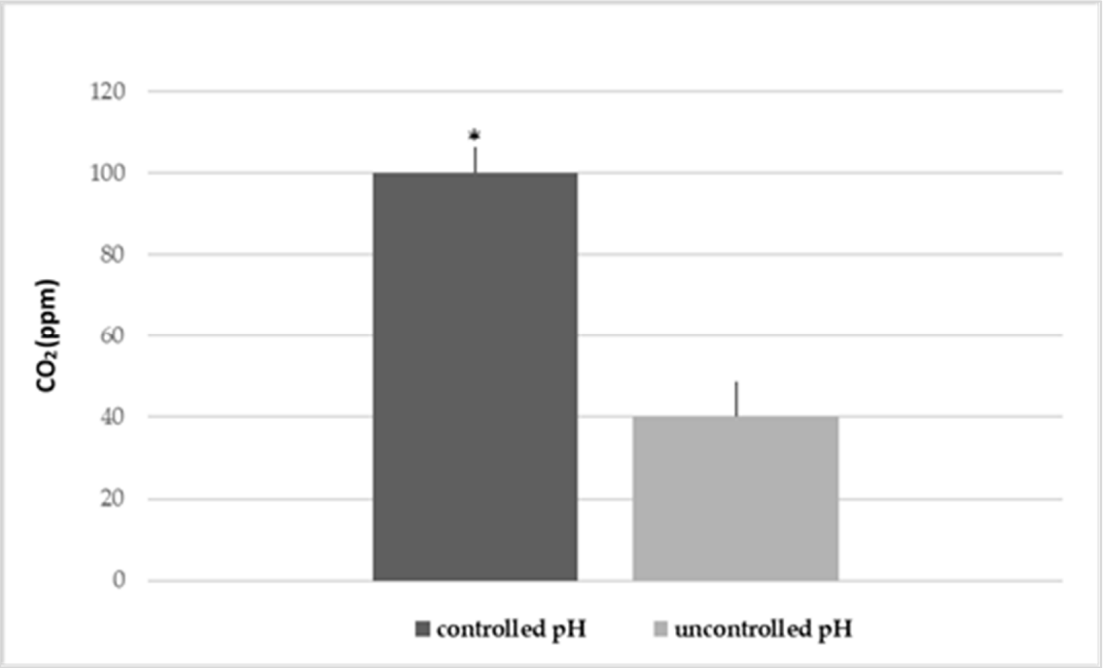

Figure 5. Mean DIC (CO_2 ppm) and standard error at the end of the three experimental weeks. Statistical significant differences ($p < 0.05$) are shown by asterisks (*).

On the other hand, although the N bio filtration efficiency is lower in uncontrolled conditions, the difference was not statistically significant, at least in the period studied (Figure 6).

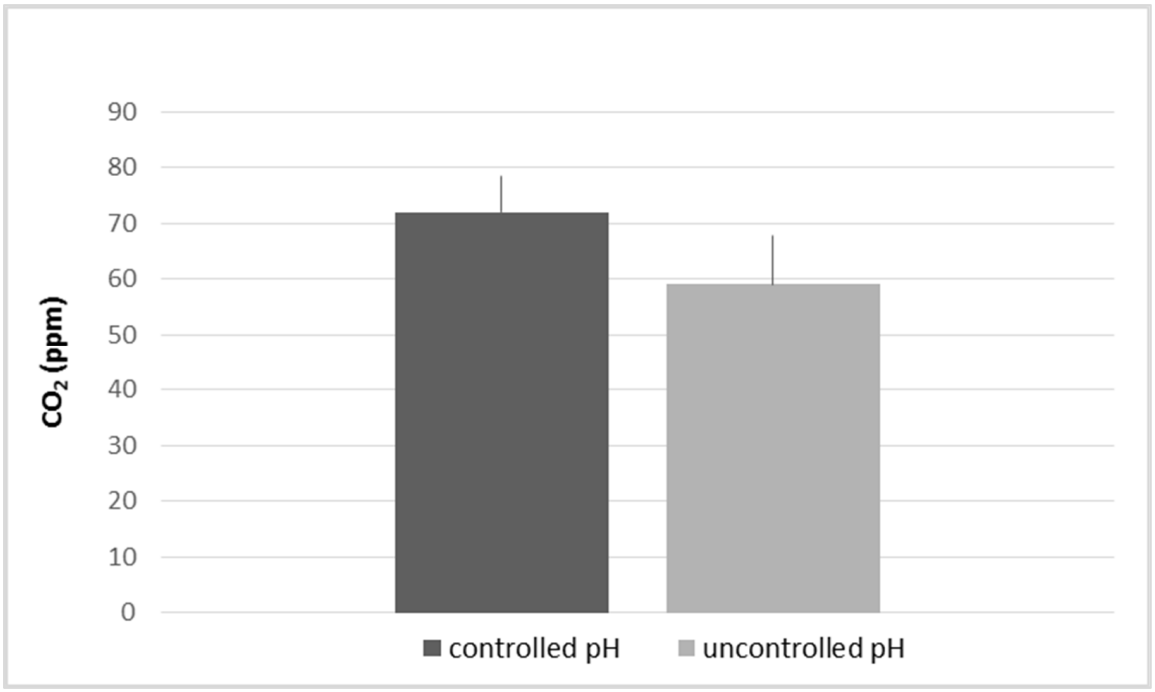

Figure 6. N bio filtration efficiency (percent) and standard error at the end of the three experimental weeks.

Elemental analysis of the biomass obtained after cultivation could only be carried out in the third week, therefore no statistical treatment could be performed. In any case, in this last week, it is noteworthy that in both conditions the C content was identical (28.8%) and the protein content was very similar and greater than 20% (20.87% in controlled pH and 22.81 % in uncontrolled pH).

Regarding the cellular morphology in superficial view of the *U. ohnoi* blades, no significant differences were observed between treatments in terms of cell size, integrity, position of the plastid or number of pyrenoids per cell, comparing the initial and final state after cultivation, in both experimental conditions (controlled and uncontrolled pH), (Figure 7).

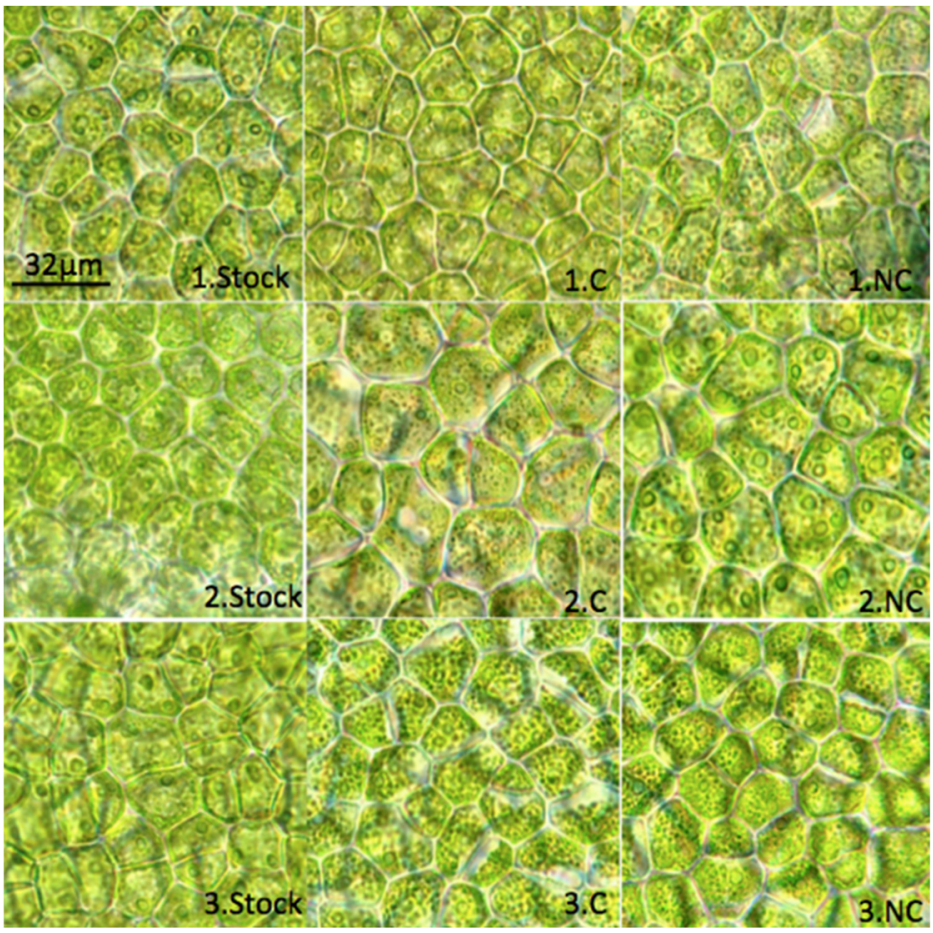

Figure 7. Cellular morphology in superficial view of the initial state and at the end of the culture period in both conditions (controlled (C), uncontrolled (NC)) in the three experiments carried out.

3.2. Influence of N, P Levels, and N: P Ratio

Figure 8 shows the contour plot of the SGR of *U. ohnoi* at the different levels of N and P. The highest growth rates (SGR > 14%; DW) were obtained with the highest levels of N and the lowest of P; while the lowest growth rates (SGR < 6%; DW) were obtained with the highest levels of P and lower levels of N.

Regarding the cellular morphology of the *U. ohnoi* thallus depending on the nutrient levels in Figure 9, it can be seen that at the lowest level of nutrients (2N/0.2P) the cells are larger and have a rather disorganized plastid, probably a consequence of oxidative stress. In the other two levels (42N/0.6P and 82N/1P) the cells are smaller and have a more structured plastid with visible pyrenoids.

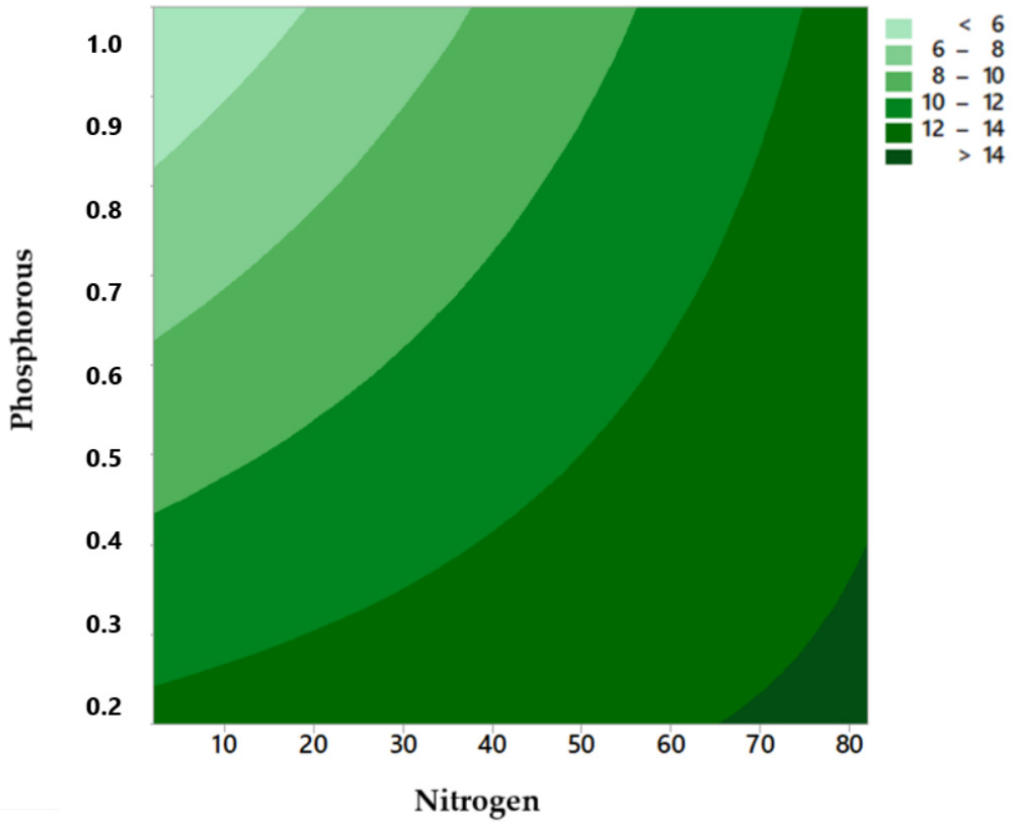

Figure 8. Contour plot of the specific growth rate (SGR) in dry weight (DW) of *U. ohnoi* at 14 days of culture after the acclimatization phase for the different ppm of N and P.

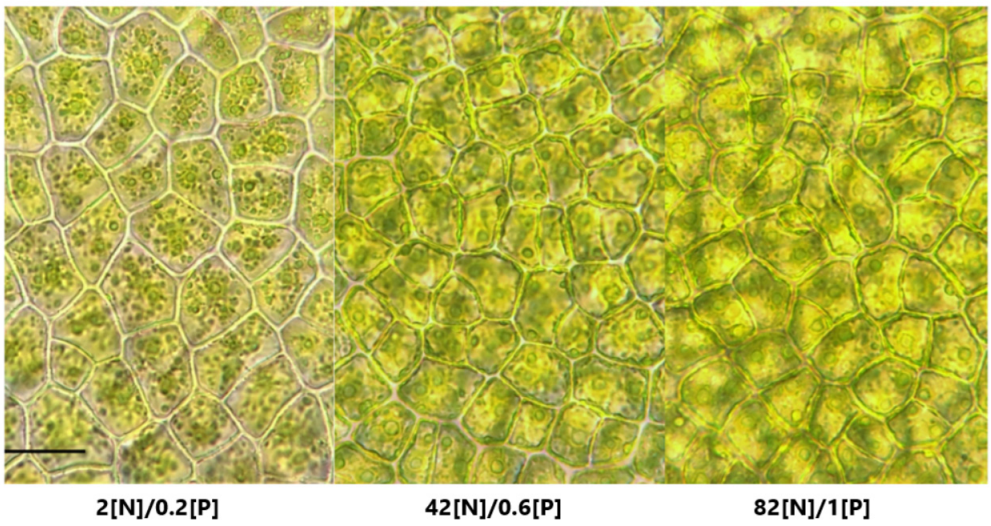

Figure 9. Superficial view of the blade of *U. ohnoi* subjected to the three different levels of nutrients (2N/0.2P, 42N/0.6P and 82N/1P). Scale bar = 25 µm.

As can be seen in Figure 10, the N bio filtration efficiency (NRE) was greater than 70% at low levels of N (2 ppm) and, obviously, much lower at higher levels (42 and 82 ppm, respectively). However, the N removal rate (NRR) increased and showed statistically significant differences between the three levels. With respect to the bio filtration efficiency and elimination rate of P (PRE and PRR), the behavior was quite similar to that of N, with the only exception that the PRE is generally higher than NRE and inversely proportional to the concentration of P.

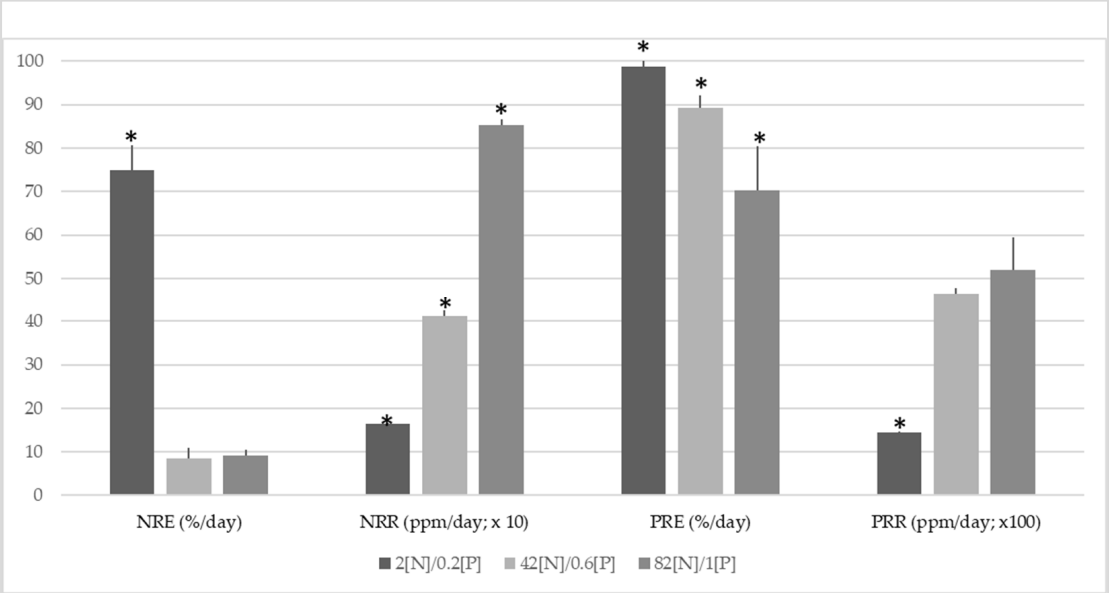

Figure 10. Bio filtration efficiency (RE) and removal rate (RR) of N and P at the different levels of these elements. Statistical significant differences ($p < 0.05$) are shown by asterisks (*).

Regarding dry matter, there were no significant differences in the different levels of nutrients (Figure 11). However, significant differences were observed in the concentration of nitrogen and, therefore, in proteins. It was observed that the highest values of proteins (above 15%) were obtained at high levels of N (42 and 82 ppm); and significantly higher at levels 42/0.6 and 82/1 with respect to level 82/0.2. Significant differences were also observed in the concentration of C, which was smaller with the lower the concentration of P. As for the percentage of H and S, very scarce significant differences were observed between all levels, the only notable being the greater amount of S at the lowest level of nutrients (2/0.2).

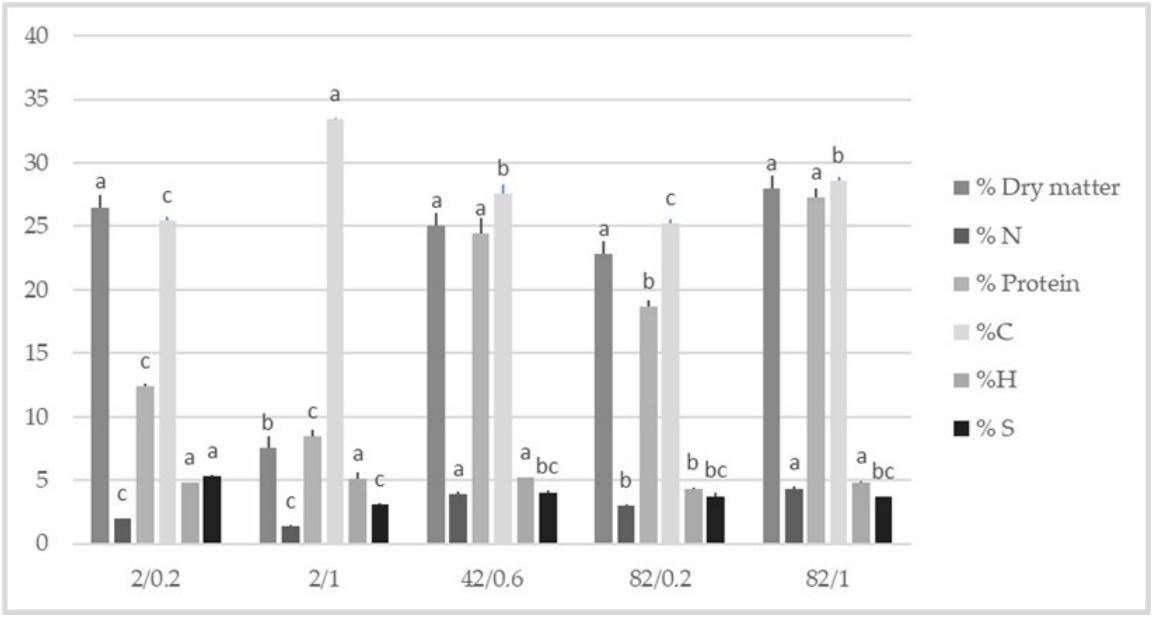

Figure 11. Percentages of dry matter and of the different elements (N, C, H, S) depending on the levels of N and P. The calculation of proteins has been carried out by multiplying the percent of N by 6.25 [35]. Different letters indicate significant diferences ($p < 0.05$).

4. Discussion

4.1. Influence of the Variations in pH and DIC

The strong variations of the pH values observed in the uncontrolled pH condition (Figure 2) are due to the photosynthetic activity and respiration of *U. ohnoi* which promotes, respectively, alkalinization or acidification of the culture medium during the periods of light and darkness. This variation is due to the fact that during photosynthesis *U. ohnoi* removes CO_2 from the medium, which causes an increase in pH. During the night, the respiration of the algae biomass releases CO_2 back into the medium, which causes a new acidification. It is observed that over time the pH range is narrower and the values higher, undoubtedly due to the gradual depletion of DIC, which is no longer capable of acting as a pH buffer. The high pH values in the uncontrolled condition in the last four days of the experiment (9.6–10.1) suggest that *U. ohnoi* was very capable of utilizing HCO_3^- from seawater as its inorganic carbon source to drive photosynthesis.

In this type of closed systems, pH control with CO_2 injection has positive effects on the productivity of the *U. ohnoi* culture. This positive effect seems to be due more to the maintenance of DIC in the culture medium than to the mitigation of pH oscillations. In the first days of culture, despite notorious differences in pH variations in the two experimental conditions (Figure 2), no differences in productivity were observed. These differences are not established until the fourth day of culture (Figure 3).

Variations in the availability of inorganic carbon can be a limiting growth factor for seaweeds. For example, in *Gracilaria conferta* (Schousboe ex Montagne) Feldmann and Feldmann observed that when a controlled treatment at pH 8 with low water exchanges was used, the algae showed the same weekly growth rate as a non-controlled pH treatment with high water exchanges. However, a non-controlled treatment with a low water exchange showed significantly decreased growth rates due to depletion of DIC [29,46]. Results of semi-enclosed water cultures of *Ulva ohnoi* in large land-based ponds [32] indicated that the gradual rise of pH can be correlated to seaweed growth. Due to the semi-enclosed water circulation in the ponds and the seaweed photosynthetic activity (i.e., consumption of HCO_3^-), pH tended to rise from week to week until reaching maximum values near 9.5 and maintaining those values, indicating that carbon could be limiting for photosynthesis. These conditions could lead to a decrease in carbon fixation and consequently, in growth efficiency, as demonstrated previously for the genus *Ulva* and other macroalgae [47–49].

According to Zou [49] in *Ulva conglobata*, a severe inorganic carbon limitation alters the performance of different photosynthetic processes (maximum quantum yield, effective quantum yield, photosynthetic conductance), lowering the energetic requirement for the HCO_3^- utilization mechanism and activating the transport of the inorganic carbon from culture medium to the algal cell as a mechanism to adapt to this limitation. The results obtained by Zou [49] in *U. conglobata* suggest that the usable energy and carbon skeleton outputs required for biosynthesis and cellular maintenance were substantially decreased in inorganic carbon-starved *U. conglobata* thalli. Similar results were obtained in our case with *U. ohnoi*. It can be seen that from the fourth day of growth, the wet weight in the uncontrolled condition is significantly lower than in the controlled condition (Figure 3), which is presumably due to the gradual lower availability of inorganic carbon that affects its growth. Likewise, there are significant differences in the growth rates in both conditions (Figure 4), although not in the efficiency of N bio filtration, at least in the period studied (Figure 5).

4.2. Influence of N, P Levels, and N: P Ratio

As already stated, in RAS systems the concentrations of N and P and their ratios are usually very different from those of the natural environment. Nitrogen forms, such as nitrate (NO_3-N), are the end product of nitrification process, the nitrogen product is less toxic for fishes, as the 96 h lethal concentration values are more than 1000 mg NO_3-N L^{-1} [33], although it is recommended that its concentration levels should be lower than 10 mg L^{-1} [50].

On the other hand, PO_4-P levels, although high in water recirculating systems with respect to the natural environment, are not considered toxic to fish; however, this element

and the N: P ratios do have a great relevance in seaweed development [27,28]. Species with fast growth, such as *Ulva* spp., cannot rely on stored P for long, and therefore their performance requires continuous availability and supply of DIP [37].

As was already stated, N: P ratio has great relevance in seaweed development [28]. For example, in *Ulva fenestrate* Postels & Ruprecht, the effect of P-limitation on growth rate was more pronounced than was N-limitation, and this could be due to a more rapid depletion of internal phosphorus pools compared to nitrogenous constituents, yielding a faster response in growth rate [51]. The results of these authors suggest that determination of N: P ratios in batch and semi continuous cultures may provide a good index to evaluate nutritional status of macroalgae. In *Gracilaria cornea* J. Agardh, the N: P ratio had a significant effect on the relative growth rate and the best one was obtained for N: P ratios from 10:1 to 10:5. The treatment with the N: P ratio of 10:0 had the lowest growth and did not differ significantly from the 10:10 ratio [52]. The reduction of growth observed in *G. cornea* with 10:10 ratio could be related to a detrimental effect of high phosphate concentrations. On the other hand, phosphate levels about 1 mM were found to inhibit growth of *Gracilaria conferta* [46], while an increase of N: P ratio (2.5–20) caused a significant growth rate enhancement [53].

In this work, similar results have been obtained in *U. ohnoi*. As can be seen in Figure 8, the highest growth rates are obtained with the highest levels of N and lower levels of P; while the lowest growth rates are obtained in the opposite scenario. However, the N and C content of biomass of *U. ohnoi* grown at the highest N and lowest P levels (82/0.2, respectively) is significantly lower than when P levels are higher (42/06 and 82/1; Figure 11), although at these levels the growth rate is somewhat lower. It is also observed that the possible negative effect on SGR of high levels of P seems to be significantly reduced at high levels of N. All these facts seem to indicate that the SGR of *U. ohnoi* is little affected by the levels of N in the cultivation environment, but it is very sensitive to P levels, which have a progressive growth-inhibiting effect, especially at low N levels, that is, at high N: P ratios.

Observing the interval of N: P ratios studied: 2 (2/1), 10 (2/0.2), 70 (42/0.6); 80 (82/1) and 410 (82/0.2) and its relationship with the SGR and nutritional composition, it seems that it is necessary for a correct *U. ohnoi* development simply to reach a minimum of N: P ratio. In the ratio 2 (2/1) we find very significant differences in the percent of carbon (very high) and the lowest values of N and, therefore, proteins (Figure 11). It could be said that N is never toxic and that P, although limiting, should not be in an N: P ratio of less than 10 to prevent its toxic effect.

Regarding the biofiltration efficiency and removal rate of N and P, it is interesting that there are no significant differences in the biofiltration efficiency of N (NRE) in the two upper levels (Figure 10), which may mean that in a scenario of no limitation of N, efficiency is constant regardless of its level. Therefore, it is obvious that it is increasing and there are markedly significant differences in the N removal rates (NRR) depending on its concentration in the culture medium.

It is noteworthy that at the same N: P ratio, but with very different levels of both elements, similar values are obtained in the chemical composition of the obtained biomass. For example, at levels 42/06 and 82/1, which are similar in their N: P ratio (70 and 80, respectively), they do not differ significantly in any element of their chemical composition (Figure 11). However, the ratio 82/0.2 (N: P ratio = 410) gives significantly lower values of N and C, which may indicate that too high N: P ratios may also have some negative effect. These N and C levels are also low in 2/0.2 and significantly lower in N than in 82/0.2, which is logical due to the low concentration of this element in the medium. This seems to indicate that, regardless of the N: P ratio, lower P levels (0.2) have a negative influence on N and C uptake. The lower N: P ratio (2/1) strongly alters the composition biomass, reaching significantly lower levels of N, S and dry matter and the highest C level, perhaps due to a higher degree of cellular hydration.

It is known that an increase of the concentration of nitrogen in the growth medium shows a positive relationship with protein content and a negative relationship with carbohydrate content and agar yield in some agarophytes as *Gracilaria tikvahiae* McLachlan [34,35]

and in *G.* c.f. *gracilis* (Stackhouse) Steentoft, L.M. Irvine & Farnham [36]. Zou [49] showed that high N-grown *Ulva conglobata* thalli exhibited higher contents of pigments and proteins and higher photosynthetic capacity than low N-grown algae. This result agrees with our results and those reported in many other studies [48,54–57]. As can be seen in Figure 11, the protein levels of *U. ohnoi* are significantly higher with 42 ppm of N in the culture medium, reaching average values of 27% in the case of 82/1.

From the morphological point of view, the cells of *U. ohnoi* cultured with 2N/0.2P level, with lower growth rates (Figure 9), are significantly larger, probably due to their lower rate of bipartition under starved/stress condition. In addition, they show plastids very disorganized, poorly pigmented and in which pyrenoids cannot be differentiated. Cells from levels 42/0.6 and 82/0.1 do not show differences in their cell size (significantly smaller due to their higher rate of bipartition) and with evident pyrenoids.

Finally, in this study the maximum specific growth rates (SGR) of *U. ohnoi* (Figures 4 and 8) were comparable or superior to previous studies: 12% d^{-1} [30]; ~16% d^{-1} [32]); 16.8% d^{-1} [1,58]. In these culture experiments, seaweed productivity was strongly dependent on culture conditions, such as irradiance, seaweed biomass density, nutrients or water flow.

5. Conclusions

The results obtained in this study indicate that pH variations do not influence the development of *Ulva ohnoi*, although growth is slowed down in closed systems due to the depletion of DIC. Therefore, in an IMTA-RAS fish–macroalgae system, the levels of DIC must be taken into account, although this is presumably not a problem due to the physiological activity of the fish contributing CO_2 to the medium and replenishing it.

Phosphorus is an essential element for the development of *Ulva ohnoi*, but an excessive level in the culture medium could block its growth, especially in conditions of low nitrogen concentration.

The results obtained in the wide range of N: P ratios tested (from 2 to 410) allows us to conclude that this ratio should not be a limiting factor for the cultivation of *Ulva ohnoi* in IMTA-RAS systems where it is normally high. We could say that NO_3-N is never toxic for *U. ohnoi* and that P, although limiting, should not be in a N: P ratio of less than 10 if we want to prevent its toxic effects.

The best strategy to follow in an IMTA-RAS fish–macroalgae system is to maintain the highest levels of recirculation rate for the algae, because under these conditions the highest rate of nitrogen elimination and crop of *Ulva ohnoi* will be obtained, with the highest values in dry matter and protein richness. This maintenance of high nitrogen values in the cultivation of *U. ohnoi* implies a high rate of water renewal, which would also guarantee the maintenance of an adequate DIC.

A special interest of these IMTA-RAS systems with macroalgae is that the algae could in the future perform the function of the current bacterial filters necessary for the reduction of ammonium produced by fish.

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