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Physiological responses and productivity of the seaweed *Ulva ohnoi* (Chlorophyta) under changing cultivation conditions in pilot large land-based ponds

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ABSTRACT

Land based intensive cultivation systems have been proposed as an ideal option for the commercial production of high value products from seaweeds. However, many cultures on *Ulva* and other seaweeds are based on relatively small-scale facilities. The high variability of culture conditions can strongly affect the physiological performance of seaweeds, but few studies examine their phenotypic plasticity by integrating critical biological descriptors, e.g. photobiology, oxidative stress, nutrient acquisition. The purpose of this study was to determine the physiological plasticity and growth of *Ulva ohnoi* during its cultivation in land-based 40 m^3 ponds. Through an entire cultivation cycle (four-weeks), photosynthesis, respiration, pigments, antioxidant capacity and nutrient content were measured. Light, temperature, pH, and dissolved inorganic nitrogen (DIN) were simultaneously monitored in seawater. Additionally, the N-uptake kinetics of *U. ohnoi* were examined in the laboratory in order to explain the efficiency of the seaweed biomass for DIN-incorporation in the ponds after fertilization. Generally, the gradual increase in seaweed density throughout the cultivation period was directly associated to a drop in light availability and dissolved inorganic carbon (i.e. higher pH) within the ponds. These changes in cultivation conditions were related to a reduction of photosynthetic capacities, nutrient content and growth of *U. ohnoi*. N-uptake kinetics of *U. ohnoi* and the behavior of DIN within the ponds after fertilization, indicated that *U. ohnoi* was able to incorporate ammonium more efficiently than nitrate, and the presence of the former likely inhibits nitrate acquisition. The understanding of the capacity of *U. ohnoi* to acclimate to the extreme changing culture conditions, could be applied to improve its productivity and chemical composition.

1. Introduction

The commercial use of the green seaweed *Ulva* is increasing worldwide, mainly driven by its nutraceutical potential and use as human food [1]. Studies proposing the cultivation of *Ulva* for human consumption, animal feed, as biofilter and even as biofuel date from the mid- 1970s and early 1980s [2,3]. The use of *Ulva* as biofilter in integrated aquaculture systems has been tested more recently with success in abalone and fish onshore farms [4,5]. On the other hand, multiple studies have shown that *Ulva* is a rich source of bioactive metabolites [6], especially of ulvan, a sulfated polysaccharide that has been demonstrated to have anticancer, antioxidant and antimicrobial activity [7,8]. It is therefore

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relevant and timely to develop culture strategies which optimize *Ulva* production and its nutritional state.

Land-based cultivation provides an ideal option for seaweeds with high value products that do not conform with off-shore culture methods. It is also the most environmentally acceptable method for the production of biomass from seaweeds that could not be economically or sustainably obtained from natural populations [9]. The intensive cultivation of seaweeds in onshore aquaculture facilities (e.g., ponds, tanks) involves significant advantages over off-shore cultures or the direct exploitation of natural populations [10,11]. Land-based cultures allow for a better control of critical biotic (e.g., herbivory, disease, inter-specific competition) and abiotic factors (e.g., light, nutrient availability), which condition the quality and productivity of seaweed biomass, or even the stability in cellular components with biotechnological applications [9,12].

Pioneer studies on the cultivation of *Chondrus crispus* (Irish Moss) as a source of carrageenan, provided the basis for the commercial cultivation of seaweeds by a method of "tumble culture". This method consists in maintaining the seaweed growing free-floating in tanks by a convective water movement caused by aeration from the bottom of the tank [10,13,14]. The resuspension of the seaweeds through aeriation avoids the formation of gradients within the tanks, and alleviates the effects of light-limitation associated to fronds self-shading as biomass increases. The exposure to "light-flecks", as a result of the movement of the seaweed from the bottom to the surface, seems to be positive for the growth and the photosynthetic productivity of *Ulva* and other species, such as *Gracilaria foliifera* and *Palmaria palmata* [15–17]. Also, the dynamic movement of seaweeds within tanks can reduce the thickness of the boundary layer over the thallus, facilitating nutrient acquisition and photosynthesis [13,16]. By contrast, potential carbon limitation for photosynthesis as the biomass increases, or the control of the temperature, are still factors difficult to control at commercial scale [2,10,13]. Low availability of CO_2 and HCO_3^- and extreme variations of temperature can drive harmful impacts on algae metabolism (e.g., photosynthesis, respiration, nutrient uptake and assimilation) and thus, their productivity [4,18,19].

For decades, the evaluation of the physiological responses of crop plants has been essential to optimize their productivity and chemical composition under stressful conditions, such as osmotic and temperature stress [20,21]. Comparatively, the knowledge available for cultivated seaweeds is much more scarce. The potential for the commercial cultivation of *Ulva* has been evaluated in different semi-enclosed culture systems, although most of the works only focused on few biological descriptors and culture physico-chemical variables [16,18,19,22,23]. Studies in *Ulva fasciata* showed how nitrogenous sources, temperature and salinity influence the growth, reproduction and chemical composition [24–26]. Different physiological aspects of cultivated macroalgae have been assessed previously [27]. Changes in the chemical composition, growth and amino acid content in *Ulva ohnoi* were examined under different light, temperature and nitrogen conditions [12,28], but the available information for seaweed cultures is still limited [1,16].

More recent studies focused on the cultivation of *Ulva* integrated aquaculture systems [4,5,23], or as raw material for the production of biofuel [29] or biomass [30], have improved the understanding of the variability of its productivity in systems approaching commercial scenarios. These previous experiences have provided valuable insights which allowed `*Ulva* domestication´in land-based ponds. However, these were rarely performed in large-volume aquaculture structures ([19,23,29–35]; see also Table S1 in Supplementary Material), which could complicate the extrapolation of their findings to larger commercial scales. Furthermore, little is known about the changing performance of the algae due to changes in the light field and carbon availability as consequence of seaweed growth. The adaptability of a plant to these changing conditions during a culture cycle needs to be determined for specific strains under open pond culture conditions, in order to maintain the algae under the log-phase of growth when seaweed productivity per unit area can be maximized.

In this study we assessed the effects of changing culture conditions (light, DIC, temperature, nutrients) on the productivity (growth, yield) and physiology of *Ulva ohnoi*. As far as we know, this is the first study to be performed in large land-based ponds at commercial-scale (40 m^3), looking at the physiological performance of the plant on a daily basis. Culture conditions and the biological performance of *U. ohnoi* were monitored during an entire four-week culture cycle. Physiological descriptors analyzed include photosynthetic rates, respiration, antioxidant capacity, proximate composition and pigment concentration. The uptake kinetics for dissolved inorganic nitrogen (DIN) of *U. ohnoi* were quantified and compared to changes in the concentration of DIN species used as fertilizer.

2. Material and methods

2.1. Evaluation of biological responses of U. ohnoi in commercial land-based ponds

2.1.1. Cultivation system

The cultivate seaweed, *Ulva ohnoi* M. Hiraoka & S. Shimada, was collected in Bahía San Quintín, B.C. (30° 30′ N, 116° W), in August 2015. A detailed description of the molecular procedures developed for the species identification was provided in the Appendix B of Supplementary Material. The culture system was constructed in 2013 within the facilities of the Autonomous University of Baja California (UABC, Instituto de Investigaciones Oceanológicas-IIO), in the city of Ensenada (Baja California, Mexico) (Fig. 1). The aquaculture unit consists of rectangular ponds (10 × 4 m), oriented to the south, each one covered with white plasticized polyvinylchloride (PVC-P); the interior walls of each pond were constructed with a slight angle towards the bottom. These characteristics were selected to optimize the incidence and dispersion of sunlight within the ponds. Each pond consists of a semi-enclosed circulation system, in which seawater quality was maintained through mechanical filtration (50 μm to 1 μm) and UV radiation. Cultivation method consists of "tumble culture" with aeration from the bottom of the pond. Each pond had an aeration system consisting of a perforated PVC tube placed longitudinally throughout the bottom, which allows for the convective movement of the water column [10,13]. Based on previous experimental trials, the culture cycle duration was 4-weeks, including an initial planting time of 1 kg FW m^{-3} (FW = fresh weight) at the first week, and a final harvest time (fourth week) when *U. ohnoi* biomass reaches about 6–7 kg FW m^{-3}. Fertilization events were done by pulses three times per week using an agricultural fertilizer (NH_4NO_3 + PO_4^{3-}). The fertilizer was added to each pond at dawn to a final concentration of 500 μM NH_4NO_3 and 50 μM PO_4^{3-}, and then (about 12 h later) ~80% of the total pond volume was discarded and replaced by new filtered seawater.

2.1.2. Experimental and sampling design

This study was carried out in early spring (March 2017), during a culture cycle consisting of four consecutive weeks (W1 to W4). Monitoring of *U. ohnoi* biomass yield in previous years indicated that this season corresponds to the peak of seaweed productivity. Three independent culture ponds were selected as experimental units ($N = 3$). The culture in each pond started with an initial biomass of ~1 kg FW m^{-3}. Sampling of the physico-chemical and biological variables was carried out in two consecutive days per week: one corresponding to the fertilization day (F), and the other corresponding to the following day, after seawater replacement (R). In each day (F or R) the biological descriptors were measured at midday, while physico-chemical variables were monitored throughout each sampling day. For the analyses of pigments, proximate composition, total phenols and antioxidant capacity, three seaweed samples were collected per culture pond. Values obtained for each biological descriptor were averaged per pond to obtain the true replicate.

Fig. 1. Land-based ponds for *U. ohnoi* cultivation at the Autonomous University of Baja California (Ensenada, Baja California, Mexico). Cultivation method consists of "tumble culture" with aeration from the bottom of the pond. Uniquely the four smaller ponds of 40 m^3 were used for the study.

2.1.3. Physico-chemical variables

Measurements of irradiance, pH, temperature, dissolved inorganic nitrogen (DIN), salinity and dissolved oxygen (DO) were made three times per day (7:00-dawn, 12:00-midday and 17:00 h-sunset). The incident irradiance (photosynthetic photon flux density) was measured with a 4π underwater spherical quantum sensor (LI-COR LI-193, Nebraska, USA), attached to a datalogger (LI-COR LI-250A, Nebraska, USA). Irradiance was measured at two depths within each culture pond: 30 cm (where measurements of seaweed photosynthesis and growth were performed, see below), and at the bottom of the pond (~ 1 m depth). Values of pH, temperature, DO and salinity were obtained by using a multiparameter probe (YSI Professional Plus, Ohio, USA).

For nutrient analyses, three samples of filtered seawater (GF/F Whatman) were collected three times a day (7:15, 12:00 and 17:00 h) per pond, except for the F days in which an additional sample was collected just before the addition of the fertilizer (7:00 h). The analysis of nutrients was performed with an automated AA3-HR (Seal Analytical) nutrient analyzer following guidelines described in the GO-SHIP Repeat Hydrography Manual [36].

2.1.4. Photosynthesis and respiration

Photosynthetic and respiration rates were measured in fronds enclosed in nine transparent polycarbonate chambers (500 mL), three chambers per pond. During the incubations, each chamber was attached to a small buoy to keep it floating free within the upper half of the water column. Short-term (20 min) incubations and a biomass/volume ratio of 0.4–0.8 g DW L^{-1} were selected to avoid potential carbon limitation [37]. Three incubations were done per pond once a day (near the zenith, 12:00 h). Photosynthesis and respiration rates were calculated from the production/consumption of dissolved oxygen within the chamber. Gross photosynthetic rates (gross-P, μmol O_2 g^{-1} DW h^{-1}) were calculated by the sum of respiration to the net photosynthesis. Respiration rates were performed in complete darkness by covering the incubation chambers with black plastic bags. Respiration rates, R (μmol O_2 g^{-1} DW h^{-1}), were calculated by measuring the reduction of DO after 60 min of incubation, while net-P was calculated by the increase in DO after 10 min. Concentration of DO (mg L^{-1}) was measured by using a polarographic sensor (YSI Professional Plus) connected to a sampling port. Values of photosynthesis and respiration obtained per pond at each sampling time were averaged to obtain the true replicate ($N = 3$).

2.1.5. Pigments

Collected seaweed fronds were kept frozen (−18 °C) until the analyses were performed. Fresh tissue (~0.1 g FW) was homogenized in a mortar with acetone (80% *v*/v) and kept in darkness at −4 °C during 24 h. Then, the extracts were centrifuged (−4 °C, 1000 rpm). Chl*a*, Chl*b* and total carotenoid concentrations were determined by spectrophotometry following the equations in Ref. [38].

2.1.6. Chlorophyll a fluorescence

The Chl*a* fluorescence of the PSII was measured by using a submersible Pulse-Amplitude-Modulated fluorometer (DIVING PAM, Walz, Germany). At the beginning of the sampling days and previous to dawn, maximum quantum yield (F_v/F_m) was measured in randomly selected fronds by exposing them to a saturating light pulse (5000 μmol photons m^{-2} s^{-1}, 0.8 s) [39]. The individual frond was held in the DCL-8 clip holder so that a constant distance between the leaf and the fiber optic was maintained. Fronds were maintained attached to the clip holder during the entire sampling day; this ensured that the subsequent measurements could be done exactly at the same site of the frond, thus avoiding intrinsic photochemical variability within each frond. Selected fronds were kept floating in the same chambers used for photosynthesis, but open at both sides. Rapid Light Curves (RLCs) were made at midday by exposing *U. ohnoi* to eight actinic light intensities (E; 18, 34, 52, 82, 147, 233, 363 and 554 μmol photons m^{-2} s^{-1}) at 20 s intervals. RLCs provided values of effective quantum yield ($\Phi_{PSII} = \Delta F/F_{m'}$), absolute

electron transport rate [ETR = ($\Delta F/F_{m'}$) · E · A · 0.5] and non-photochemical quenching [NPQ = ($F_m - F_{m'}$) / $F_{m'}$] [40,41]. The thallus absorptance (A) was calculated according to the procedures and equations [42], and based on the method in Ref. [43].

2.1.7. Proximate composition

The proximate composition of fronds was determined following the A.O.A.C. methods (2005) [44]. After collection, samples were oven-dried at 60 °C until constant weight and pulverized to a fine powder. The nitrogen content (% DW) was determined by the micro-Kjendahl method. The percentage of protein was calculated by multiplying the N-content by a factor five [45]. Soluble carbohydrates (% DW) were quantified by the phenol-sulfuric acid method ([46], modified in Ref. [47]). Briefly, dried-ground tissues were hydrolyzed with 0.2 M HCl at 60 °C during 3 h, and centrifuged. An aliquot of supernatant was mixed with distilled water, 3% phenol and concentrated sulfuric acid. After 30 min, absorbance was determined at 490 nm in spectrophotometer, using ramnose as calibration standard. Ash content was obtained from dried samples, incinerated in a muffle furnace at 500 °C during 4 h; ash content (% DW) was determined by weight difference between incinerated and dried samples. Total lipids (crude fat) were determined by extraction with petroleum ether for 6 h in a Soxhlet system.

2.1.8. Total phenolic content and antioxidant capacity

Collected fronds were dried, ground and extracted in aqueous methanol 80% in darkness for 24 h. After centrifugation, the phenolic compounds and antioxidant capacity were quantified in the methanolic supernatants. Phenolic compounds were measured by the Folin–Ciocalteu assay [48] with gallic acid used as a standard. A volume of methanolic extract was diluted in distilled water. Then, 0.1 mL of Folin–Ciocalteu reagent and 0.3 mL of distilled water saturated in $NaCO_3$ were added. The mixture was homogenized, heated (40 °C for 3 min) and absorbance was measured at 765 nm with a spectrophotometer. The radical scavenging activity of the same methanolic extracts was also determined [49]; the reaction mixture was prepared with 0.1 mL of diluted extract (1:4 with aqueous methanol at 80%) and 1 mL of DPPH 30 μM dissolved in aqueous methanol (90%). Absorbance at 517 nm was read after 30 min of DPPH addition. The total antioxidant capacity of algal extract was expressed as ascorbic acid equivalents.

2.1.9. Growth

Ulva ohnoi growth was quantified in fronds (0.5 g FW) kept within transparent polycarbonate chambers (500 mL) floating freely in each pond (i.e. three chambers per pond, and two fronds in each chamber). The incubation chambers were open at both sides to ensure an appropriate water exchange; plastic fiber mesh covered these openings to keep the fronds within the chamber. The change in fronds weight was measured every week. The specific growth rate (SGR) was calculated with the following equation [50]:

$$\mathrm{SGR}\left(\%\mathrm{FW\ day}^{-1}\right) = 100 \times 1\left[(ln\ W_t - ln\ W_0)/t\right]$$

where W_0 and W_t are the fronds weight at the beginning and at the end of the growth period (t, days). The biomass yield of the culture was determined with the following equation:

$$\mathrm{Y}\left(\mathrm{g\ FW\ m}^{-2}\ \mathrm{day}^{-1}\right) = \left(W_f - W_i\right)/t$$

where W_f and W_i were the total biomass (g FW m^{-2}) within the ponds at the end and at the beginning of each week (W1 to W4), and t is the time in days.

2.2. DIN uptake kinetics

In laboratory, *U. ohnoi* fronds collected from the ponds in the middle of the cultivation period (i.e., week 2) were separately incubated with increasing concentrations (25, 50, 100, 200, 400 and 600 μM) of labelled $^{15}NH_4$ Cl and $^{15}KNO_3$ (at.% = 99, Cambridge Isotope Laboratories) in 500 mL transparent polycarbonate chambers. Three replicates were made for each nutrient concentration. During the incubations, the chambers were placed in large incubators (2015 VWR® Signature ™) at constant temperature and light conditions corresponding to the average values within the ponds in the second week, i.e., 16 °C and 250 μmol photon m^{-2} s^{-1}. The chambers were constantly agitated to minimize the effect of the boundary layer on nutrient acquisition, and the formation of nutrient gradients [51]. Following previous trials, an algae biomass per seawater volume of about 0.1–0.2 g FW L^{-1}, and incubations of 30 min, were selected to avoid substantial changes in seawater nutrient concentration as well as carbon limitation. After the incubations, the fronds were rinsed with deionized water and dried at 60 °C for 48 h. Finally, the samples were pulverized to a fine powder and encapsulated in tin capsules. Isotopic determinations were carried out at the UC-Davis Stable Isotope Facility using an EA interfaced to a continuous flow IRMS. The incorporation of ammonium and nitrate (V, expressed as μmol N g^{-1} DW h^{-1}) was calculated as:

$$V = \left[\left({}^{15}N_{exp} - {}^{15}N_{back}\right) \times N_C\right]/(M_N \cdot t)$$

where the difference ($^{15}N_{exp}$ – $^{15}N_{back}$, at.%) is the ^{15}N enrichment relative to ^{15}N in fronds non-exposed to labelled nutrients (i.e., background ^{15}N), N_C is the nitrogen content (g N g^{-1} DW), M_N is the molar mass of N, and t is the duration of the incubation. The incorporation rates (V) were plotted against the substrate concentration (S, μM) and the uptake rate parameters were calculated with the Michaelis-Menten model:

$$V = (V_{max} \cdot S)/(K_m + S)$$

where V_{max} is the maximum incorporation rate (μmol N g^{-1} DW h^{-1}) and K_m is the saturation constant (μM); V_{max} and K_m were estimated by graph analysis in SigmaPlot 11 program (Systat Software Inc). Affinity constant α was calculated as V_{max} / K_m.

2.3. Statistical analysis

The general biological responses of *U. ohnoi* with the changing culture conditions were analyzed by multivariate approaches using PRIMER 6 and PERMANOVA+ v.1.0.2 [52]. The multivariate analyses were performed with normalized data of all response variables, and a ranked triangular similarity matrix was constructed using Euclidean distances. To visualize multivariate patterns, a non-metric multidimensional scaling (MDS) ordination was used (Fig. S1, Supplementary material). The PERMANOVA analysis (9999 permutations) had two factors with nested design, where the "day" random factor of F and R was nested within the fixed factor "week" (Table 1). Significant differences in the pair-wise a posteriori comparisons were checked using Monte Carlo P-values, due to the restricted number of possible permutations. A nested ANOVA was also applied, where the variation of the physiological responses of *U. ohnoi* within the random factor "days" (F and R) were nested within the fixed factor "weeks" (Table S1, Supplementary Material, Appendix A); a post hoc test (Student-Newman-Keuls, SNK) was run. A non-parametric Kruskal-Wallis test was used when data did not meet the assumptions of normality and homoscedasticity. A repeated measures ANOVA was applied to analyze the changes in growth and biomass yield during the cultivation period. Statistical analysis was performed with the SPSS v.20 program (SPSS, Chicago, IL, USA). Raw data for the different variables are shown in Table S3 of Supplementary Material (Appendix A).

Table 1
Results of the nested PERMANOVA performed to analyze the physiological changes of *U. ohnoi* throughout the cultivation period (weeks; W1, W2, W3, W4) and the sampling days (F: fertilization, R: water-replacement).

Main test	SS	MS	Pseudo-F	*P* (perm)
Week[a]	259.29	86.431	5.0553	**0.0021**
Day (Week)	68.389	17.097	4.3203	**0.0001**
Pair-wise a	t	P (MC)		
W1 × W2	1.7112	0.1012		
W1 × W3	2.7969	**0.0207**		
W1 × W4	4.0488	**0.0085**		
W2 × W3	1.4061	0.1738		
W2 × W4	2.0593	**0.0467**		
W3 × W4	1.4214	0.164		
Pair-wise b	t	P (MC)		
F × R (W1)	2.2207	**0.023**		
F × R (W2)	2.3345	**0.0222**		
F × R (W3)	0.1013	**0.0356**		
F × R (W4)	0.0998	0.1972		

Bold numbers indicate significant differences.
[a] P (MC) 105 permutations.

3. Results

3.1. Evaluation of biological responses of U. ohnoi

Irradiance was gradually reduced from W1 to W4 (Fig. 2A), and it decreased to values near zero from W3, both in the middle and at the bottom of the water column (Fig. 2A, B). The seawater temperature showed daily variations of up to 3 and 4 °C (Fig. 2B). The lowest temperature values were recorded during the first 2 weeks of culture (13–18 °C), while the highest were found in W3 (17–21 °C) and W4 (16–20 °C). Daily pH variations showed an increase from sunrise to sunset (Fig. 2D). In W1, pH varied from 8 to 9 while in W2, W3 and W4, pH values measured at sunrise, midday and sunset were higher than the corresponding values in W1, being as high as 9.3–10 at sunset. Salinity remained almost constant (33–33.5‰) throughout the study.

The nested PERMANOVA analysis (Table 1) showed significant changes of the physiological status of *U. ohnoi* among weeks, and between days (F and R) within each week. The higher differences were found between W1 and W4 ($P = 0.0085$), and W1–W3 ($P = 0.02$). No significant differences were detected between consecutive weeks (W1–W2, W2–W3, W3–W4). These changes were also represented graphically by MDS (Fig. S1, Supplementary Material, Appendix A).

Generally, gross-photosynthesis of *U. ohnoi* (Fig. 3A) was notably reduced throughout the culture period, being 63% lower in W4 compared to W1; gross-P showed a negative correlation with cultivation time ($R^2 = -0.74$; $P < 0.001$, Table S1, Supplementary material). By contrast, in W2 and W3, higher values (36–44%; $P < 0.001$) of photosynthetic rates were detected in the days of fertilization (F) with respect to the days of replacement of the water (R). Respiration rates remained almost constant throughout the culture cycle, except for a significant increase in day F respect to R in W2 ($H = 11.44$; $P = 0.010$, Table S1, Supplementary material) (Fig. 3B). There was a significant increase in Chl *a* content (107%; $P = 0.026$), Chl *b* (139%: $P = 0.014$) and carotenoids (27%; $P = 0.006$) in *U. ohnoi* from W1 to W4 (Fig. 3C-E, Table S1, Supplementary material). Also, Chl *b:a* molar ratio showed a positive linear correlation with cultivation time ($R^2 = 0.76$; $P = 0.002$), and it increased by ~10% from W1 to W4 (Fig. 3F).

Maximum quantum yield, F_v/F_m, started at low values (~0.7) in the first experimental day, i.e. F, W1 ($P \leq 0.001$, Fig. 4A); however, F_v/F_m rapidly recovered and increased by ~10% on the second day (R, W1). In the subsequent weeks (W2 to W4), F_v/F_m gradually decreased from 0.77 to 0.73 ($R^2 = -0.79$; $P \leq 0.001$; Table S1, Supplementary material). A general significant reduction in both ETR_{max} ($P = 0.015$) and Φ_{PSII} ($P = 0.003$) was found during cultivation, being reduced to ~50% in W4 (Figs. 4B, D; Table S1, Supplementary material). However, ETR_{max} was significantly higher in R than in F days, within W2 and W3. Significant higher values of NPQ were detected in W1 compared to the rest of the culture weeks (~40%; $P = 0.041$) (Fig. 4C; Table S1, Supplementary). Absorption by *U. ohnoi* tissues increased from 0.41 to 0.52 from W1 to

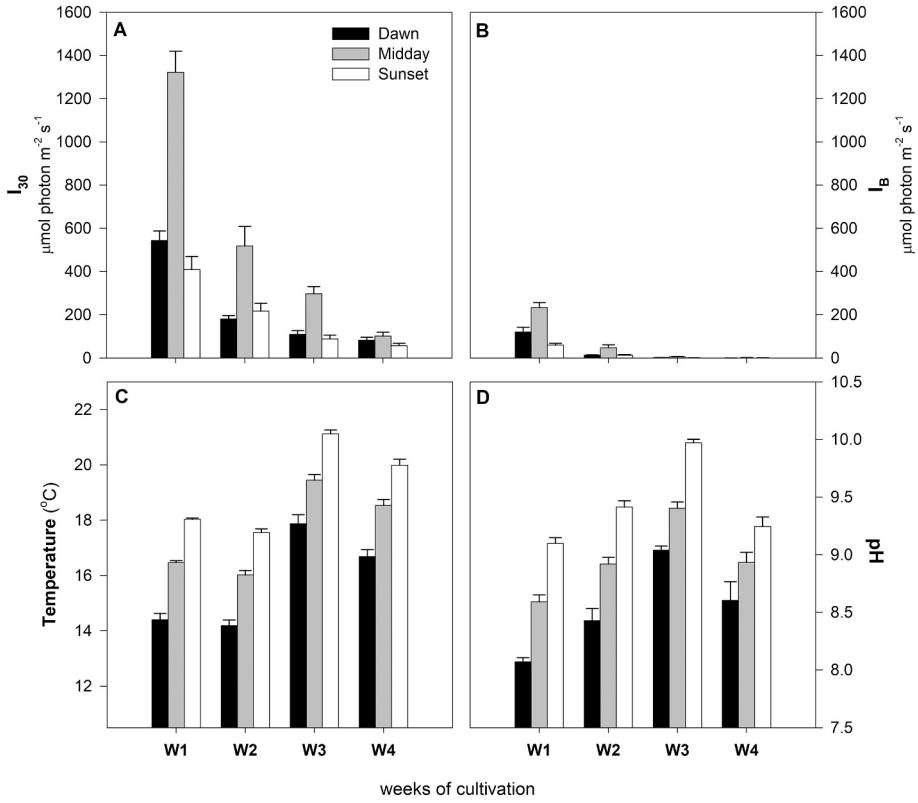

Fig. 2. Physico-chemical parameters (irradiance-A, B; Temperature-C; pH-D) measured in the culture ponds of *U. ohnoi* at dawn (black), midday (gray) and sunset (white). Values of I_{30} (panel A) and I_B (panel B) corresponded to irradiance values measured at 30 cm depth (i.e., where photosynthetic measurements were made) and at the bottom (~1 m depth) of the ponds, respectively. For simplicity, values of each factor made on each sampling day (F: fertilization, R: water-replacement) were averaged for each week. Values are means ($N = 6$) and standard errors.

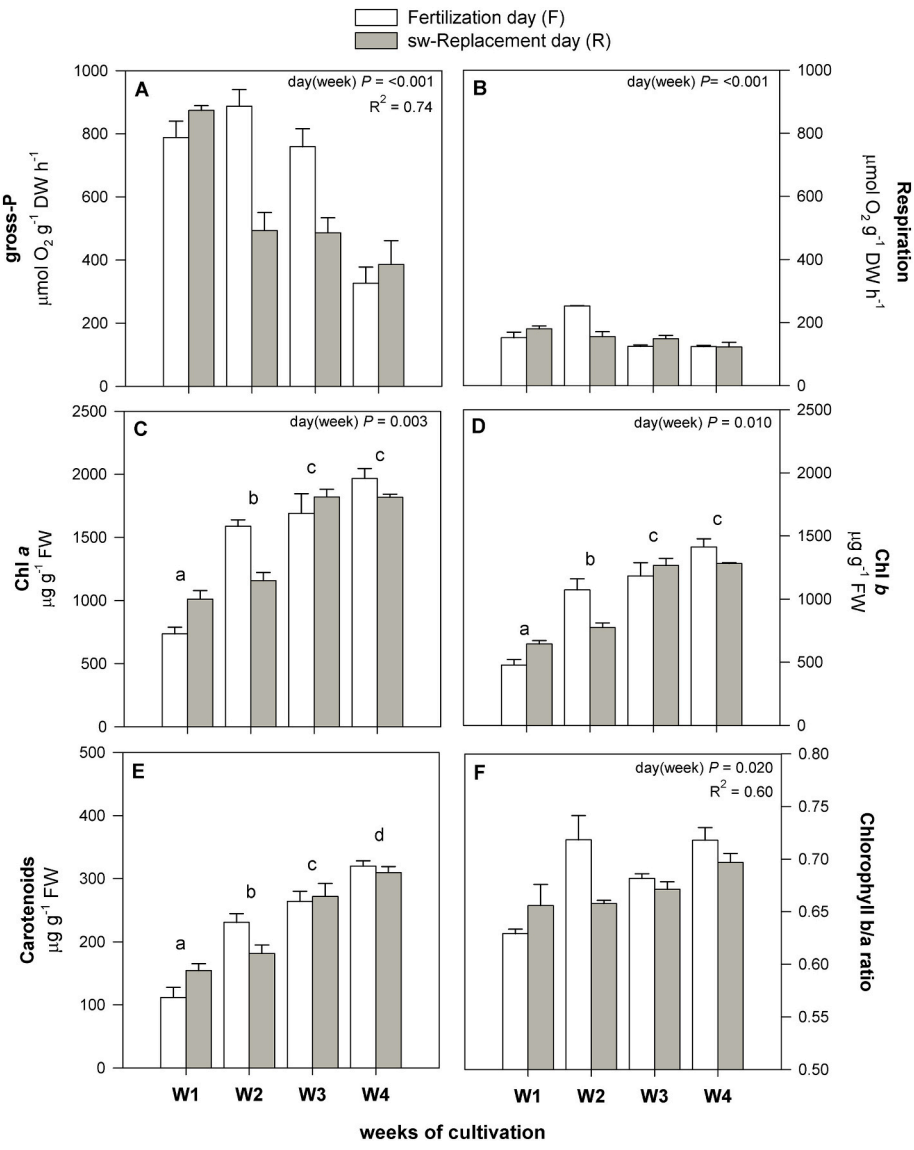

Fig. 3. Values of gross-photosynthesis (gross-P) (A), Respiration (B), Chlorophyll a (C) Chlorophyll b (D), Carotenoids (E) and Chlorophyll b/a molar ratio (F) values of *U. ohnoi* during the 4 weeks of cultivation (W1–W4), in fertilization (white) and seawater-replacement (gray) days. Different letters are used to show significant differences among weeks (nested-ANOVA, *post-hoc* SNK). Significant differences between sampling days nested within the weeks are also indicated in the right corner of the panels. When significant differences were not found among weeks by nested-ANOVA, a regression analysis was performed and R^2 was indicated. Values are means (n = 3) and standard errors.

W4, however, this increase was not statistically significant (Table S1, Supplementary material).

The carbohydrate content was significantly reduced from the beginning of the cultivation ($R^2 = 0.82$; $P < 0.001$), reaching a total decrease of 30% in W4 (Fig. 5A). Similarly, the nitrogen and protein content decreased significantly from W1 to W4 ($R^2 = 0.080$; $P < 0.001$) (Fig. 5B). On the contrary, ash significantly increased throughout the cultivation period while lipid content remained relatively constant (Fig. 5C,D) (Table S1, Supplementary material). In some weeks, content in carbohydrates, nitrogen and ashes was significantly different between F and R days.

The total antioxidant capacity (Fig. 6A) decreased significantly from W1 to W4 (~30%; $P < 0.001$, Table S1, Supplementary material). The total phenolic content exhibited a similar pattern, but only for the comparison among R days during the cultivation period (Fig. 6B).

Despite the total *U. ohnoi* biomass increased throughout the cultivation period (from ~1 to 6–7 kg FW m^{-2}), the productivity efficiency (yield) was significantly reduced by ~40% in W3 and W4 (Fig. 7A), and the specific growth drastically decreased (~80%, $P < 0.001$) from W2 (Fig. 7B).

Within each week, on F days, ammonium concentration decreased rapidly (minutes to hours) after fertilization at a concentration of ~200 µM (Fig. 8A); this reduction was faster as cultivation period advanced, and ammonium was totally consumed at the end of the day from W3. On the contrary, nitrate concentration did not decrease substantially after fertilization (Fig. 8B), and therefore, higher nitrate concentration (50–100 µM) remained within the ponds even after seawater replacement. Consumption of ammonium in seawater (i.e., removal from seawater) was significantly greater than consumption of nitrate (Fig. 8D); also, the former increased (~25%) during cultivation, while nitrate consumption decreased by ~70%. Phosphate concentration ranges were between 30.7 and 16.6 µM in F days, and 7.4–2.6 µM in R days. The reduction of phosphate concentration during F days (i.e., consumed by the seaweed biomass) was higher as cultivation progressed from W1 (40%) to W4 (62%).

Uptake kinetics of NH_4^+ and NO_3^- by *U. ohnoi* adjusted to the Michaelis-Menten model ($R^2 > 0.94$), but they were significantly different between them (Fig. 8C). Values of V_{max} were 5.5-fold higher for NH_4^+ than for NO_3^-, while K_m was 31-fold higher for NH_4^+.

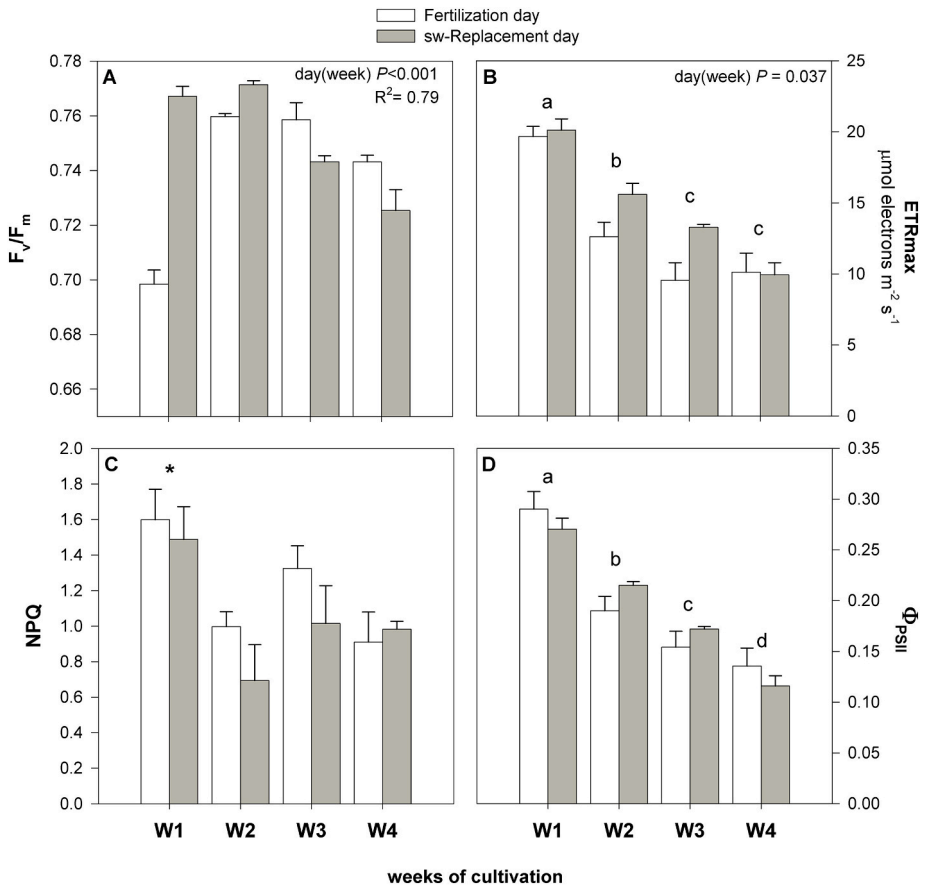

Fig. 4. Values of photochemical descriptors (maximum quantum yield-A, maximum electron transport rate-B, non-photochemical quenching-C, effective quantum yield-D) measured in *U. ohnoi* during the 4 weeks of cultivation (W1–W4), on fertilization (white) and seawater-replacement (gray) days. Values are means ($n = 3$) and standard errors. See Fig. 3 for further information on symbols and statistical analysis.

4. Discussion

4.1. Physiological responses and growth of U. ohnoi under changing cultivation conditions

During its cultivation of 4 weeks in commercial land-based ponds, *U. ohnoi* significantly increased its biomass from ~1 to ~7 kg FW m^{-3}. This biomass increase caused gradual (but severe) alterations in the culture conditions, such as carbon limitation and the reduction in light penetration in the water column of the ponds (Fig. 1), which in turn, significantly affected the physiological performance (Table 1) and growth efficiency of the seaweed (Fig. 7).

In general, photosynthetic rates of *U. ohnoi* (gross-P and ETR_{max}) decreased during the culture. This reduction may be mostly explained by the drop of available irradiance in the water column due to the increase in seaweed biomass density, and therefore, fronds self-shading. In fact, light dramatically diminished within the ponds from the second week of cultivation. Quantum efficiency (Φ_{PSII}, F_v/F_m) was also gradually reduced from W1 to W4, reflecting a diminished capacity (and/or efficiency) of the photosynthetic apparatus at thylakoid level to process light [41]. In this sense, the accumulation of photosynthetic pigments and the increase in Chl*b*/*a* molar ratio (i.e., antenna size) exhibited by *U. ohnoi* through the course of the culture can be interpreted as photoacclimation strategies to counteract light scarcity and diminished photosynthetic activity [30,53,55,58]. Furthermore, values of NPQ in *U. ohnoi* decreased from W1 to W4, because the lower the light availability the lower the need to activate photoprotective mechanisms [56,57]. The reduction of total phenolic content and antioxidant activity in *U. ohnoi* were consistent with the decreasing light availability and NPQ. Some phenolic compounds are associated to the non-enzymatic antioxidant activity that scavenges reactive oxygen species (ROS) produced by seaweeds under stressful conditions [58]; because the over-energization of the photosynthetic apparatus and ROS production are highly unlikely under low light, antioxidant activity is less required.

Contrary to the general decline of photosynthetic rates, gross-*P* values remained elevated until the third week of cultivation, but uniquely in days when fertilization took place. This suggested a positive effect of nitrogen availability on the photosynthetic activity of *U. ohnoi*, which could counteract light-limiting conditions [31,59,60]. Positive correlations between nitrogen availability and photosynthesis have also been reported in this species and other marine macrophytes [31,59,60]. Particularly in the genus *Ulva*, [54] demonstrated that *Ulva lactuca* increased its photosynthetic rates few hours after fertilization during in situ incubations. Also, N-addition can enhance photosynthetic capacities in *Ulva conglobate* [61], as well as in other seaweeds and *Ulva* species under cultivation [62,63].

In addition to low light availability, the rise in pH can be correlated to the growing seaweed biomass during the culture. Due to the semi-enclosed water circulation in the ponds and the seaweed photosynthetic activity (i.e., consumption of HCO_3^-; [41,64]) pH increased within days, but also tended to rise from week to week until reaching maximum values near 9.5 in W3. These values indicated that carbon can be limiting for photosynthesis, a condition which could additionally contribute to the gradual decrease of the photosynthetic productivity of *U. ohnoi* [65,66].

Reserve carbohydrates and total N-content (and proteins) also significantly decreased in *U. ohnoi* during cultivation. Likely, the decrease in photosynthetic productivity led to a reduction of

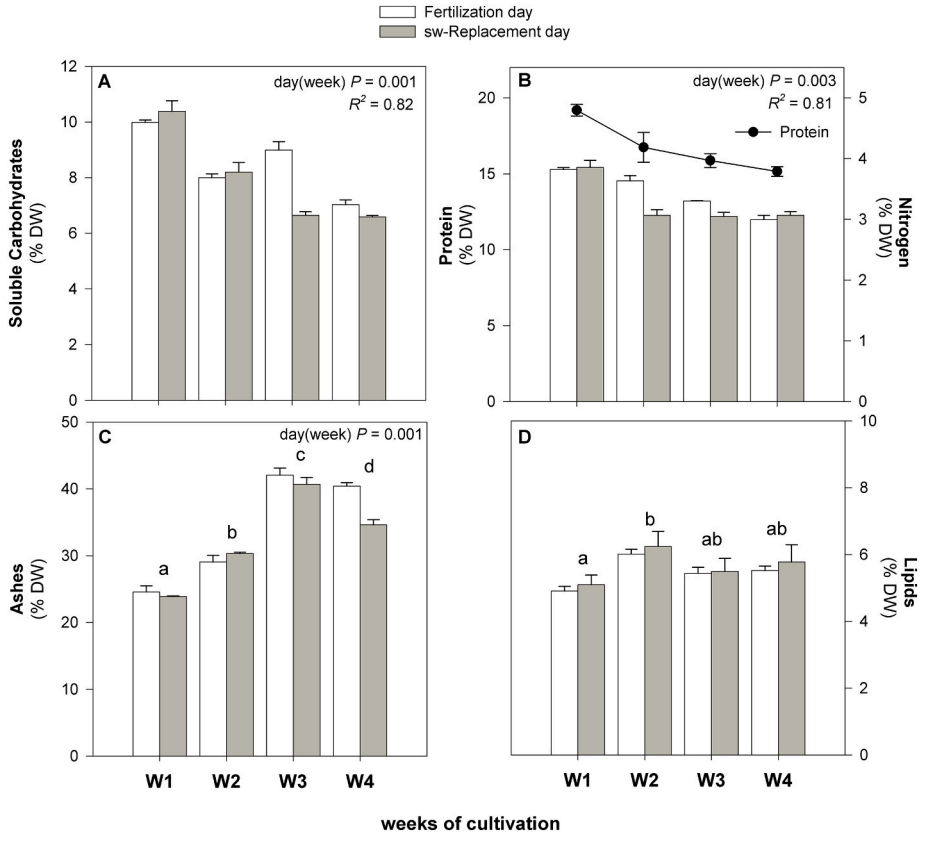

Fig. 5. Values of non-structural carbohydrates (A), total nitrogen and protein (B), ashes (C) and lipids (D) measured in *U. ohnoi* during the 4 weeks of cultivation (W1–W4), on fertilization (white) and seawater-replacement (gray) days. Values are means (n = 3) and standard errors. See Fig. 3 for further information on symbols and statistical analysis.

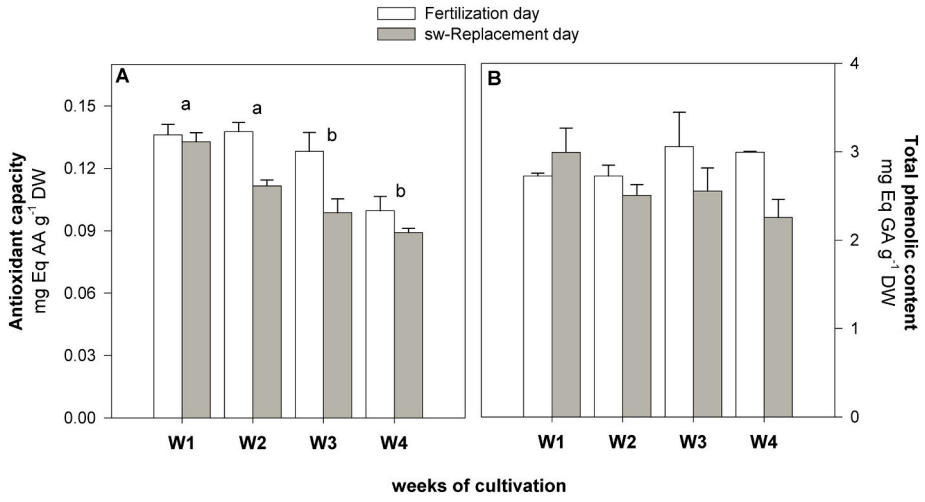

Fig. 6. Values of non-structural carbohydrates (A), total nitrogen and protein (B), ashes (C) and lipids (D) measured in *U. ohnoi* during the 4 weeks of cultivation (W1–W4), on fertilization (white) and seawater-replacement (gray) days. Values are means (n = 3) and standard errors. See Fig. 3 for further information on symbols and statistical analysis.

photoassimilates and metabolic energy, essential to incorporate inorganic nitrogen into organic compounds, such as free amino acids and proteins [27,67]. Under an "energy crisis" condition associated to light limitation, seaweeds´ internal resources can be diverted to photoaclimation responses [67], such as the synthesis of photosynthetic pigments observed for *U. ohnoi* in this study. Despite being reduced, values of N-content in *U. ohnoi* (~3.5 in W1 to ~3% DW in W4) were always well-above the critical values of 1.5–2% DW indicative of N-limitation [67]. On the other hand, the response of ashes content contrasted with N and carbohydrates, since they increased in seaweed tissues throughout the cultivation period; this opposite pattern between mineral and organic compounds content in relation to light availability has been widely described in seaweeds, as for instance in *Ulva fasciata* under cultivation [68]. Although lipid content (as well as fatty acid composition) can be altered by factors such as light and temperature in *Ulva* genera [69], this variable did not vary substantially during cultivation in

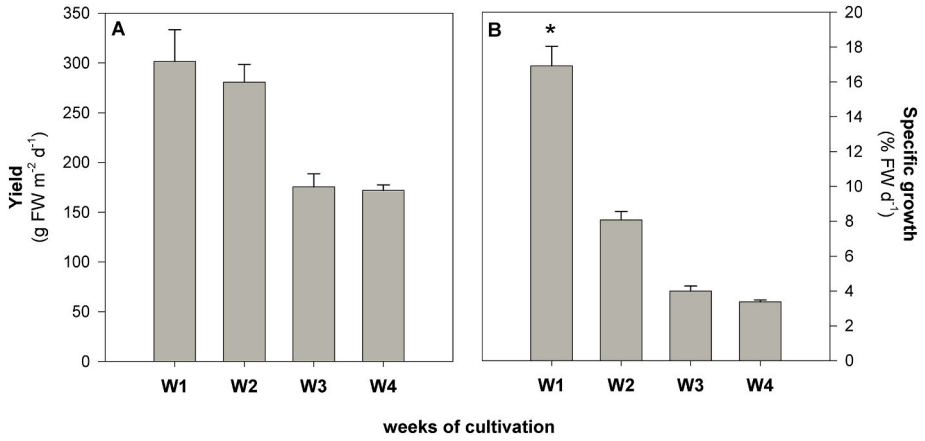

Fig. 7. Specific growth (A) and yield (B) of *U. ohnoi* during the weeks of the culture (W1–4). The values are the means (n = 3) and standard errors. The significant differences (repeated measures ANOVA and post hoc SNK analysis) between treatments are represented with an asterisk.

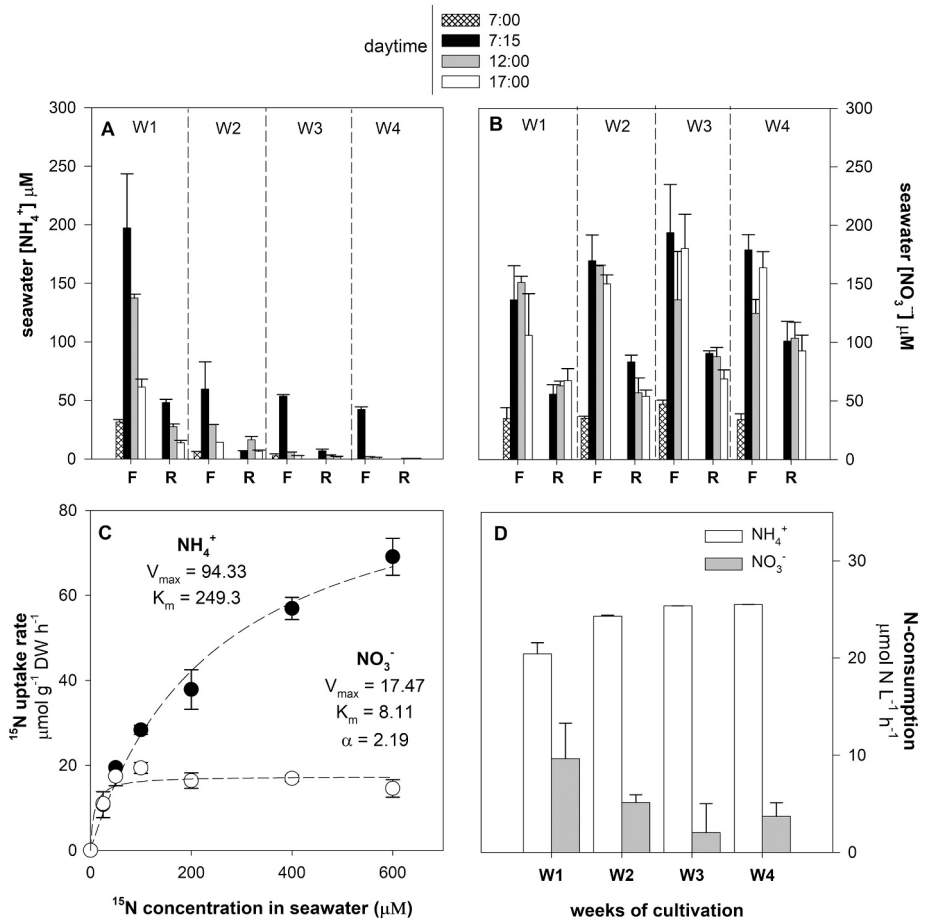

Fig. 8. Concentration of NH_4^+ (A) and NO_3^- (B) in the seawater of the ponds during the weeks of cultivation (W1–4), in fertilization (F) and seawater-replacement (R) days; the different bars indicate the time of day at which the sampling was performed. (C) Uptake kinetics for NO_3^- and NH_4^+ and derived parameters measured in *U. ohnoi* (D) Nitrate and ammonium consumption within the ponds. The values are means and standard errors.

this work.

Maximum specific growth rates (SGR) and biomass yield of *U. ohnoi* in this study (~16% d^{-1}, ~300 g FW m^{-2} d^{-1}) were comparable, or even higher, to those quantified for the genus *Ulva* in land-based culture systems in Israel (16.8% d^{-1}, 320 g FW m^{-2} d^{-1}; [4]), Denmark (16.8% d^{-1}, 433 g FW m^{-2} d^{-1}; [29]), Saudi Arabia (12% d^{-1}, 300 g FW m^{-2} d^{-1}; [19], or Australia (200 g FW m^{-2} d^{-1}; [12]). In these culture experiences, seaweed productivity was strongly dependent on culture conditions, such as irradiance, seaweed biomass density, nutrients or water flow. In this work, a reduction of SGR and yield of *U. ohnoi* were remarkable from the second and third weeks of cultivation, respectively. This could be attributed to the general reduction of its physiological performance as a consequence of limitation stressors, i.e., low light and DIC depletion. 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 [61,65,70].

4.2. DIN-uptake by U. ohnoi after fertilization

Changes in DIN species concentration after fertilization throughout the experimental cultivation clearly showed a differential utilization of ammonium and nitrate by *U. ohnoi*. Specifically, ammonium was consumed efficiently after fertilization pulses. Ammonium consumption increased as the algae biomass grew from W1 to W4 (see Fig. 8D), being totally removed from seawater within hours after fertilization from W3 (Fig. 8A). The uptake kinetic for ammonium (V_{max} = 95 μmol N g^{-1} DW h^{-1}, K_m = 250 μM) explained this consumption pattern, because it allowed its rapid (and unsaturated) incorporation by *U. ohnoi*. This rapid and highly-efficient incorporation at elevated concentration of ammonium (also known as "surge uptake") has been documented in *U. lactuca* and seagrasses during natural fertilization pulses (e.g., sediment resuspension [71–73]). Since the pK_a for $NH_3/NH_4 \approx 9.3$, the decrease in ammonium at elevated pH levels within the ponds can also respond to both the uptake of NH_3 by passive transport/diffusion, or its partial release to the atmosphere as NH_3 gas [67].

The behavior of nitrate after fertilization strongly contrasted to that observed for ammonium, since its concentration did not decrease during F days and hence, concentrations of this nutrient remained elevated even when seawater was replaced, i.e. 50–100 μM in R days. This can be partially explained by the reduced capacities to acquire this nutrient (V_{max} = 17 μmol N g^{-1} DW h^{-1}, K_m = 8 μM) compared to those for ammonium, which could be saturated during fertilization. This differential acquisition of DIN species has been well described for the genus *Ulva* [74–77] and other seaweeds [78–81], and explained by the different trans-membrane pathways to be incorporated, as well as by the metabolic energy required for their assimilation [79,81,82]. Nevertheless, and following theoretical uptake kinetics for nitrate, concentrations above 8 μM would be able to saturate its uptake at rates of ~15 μmol nitrate g^{-1} DW h^{-1}; then, it could be expected that total nitrate present in each pond after fertilization (8–10 mol in 40 m^3) should be consumed at rates between 0.12 and 0.72 mol per hour by the total seaweed biomass in each pond, i.e., ~8 to ~48 Kg DW in W1 and W4, respectively. Therefore, the absence of a substantial consumption of this nutrient cannot be fully explained solely by the reduced capacities for its acquisition, but also by a potential nitrate uptake inhibition fueled by the presence of ammonium and/or its accumulation within the cell, as demonstrated for *Ulva* and other species [76,81,83,84]. This was additionally supported by the decrease in nitrate consumption as biomass increased during the cultivation period (Fig. 8D). Furthermore, high pH and temperatures reached in seawater within the ponds in this study could contribute to the reduction in nitrate acquisition by *U. ohnoi*. The detrimental effect of elevated temperature and pH on nitrate uptake has been associated to a decrease of the nitrate reductase activity in the genus *Ulva* and other seaweeds [85–87].

5. Conclusions

This study is the first to provide the plasticity of the physiological performance of *U. ohnoi* during its cultivation in land-based ponds at commercial scale (40 m^3). Photosynthetic productivity, photoprotective mechanisms, antioxidant capacity and proximal composition of *U. ohnoi* gradually varied as cultivation conditions change. Depending on the stage of the culture, seaweed productivity (yield, specific growth) can be compromised because of light scarcity, and inorganic carbon can be limiting for photosynthesis as seaweed biomass grows. Based on the seaweed DIN-uptake kinetics and on the changes of DIN concentration in seawater after fertilization, our results also indicated that *U. ohnoi* acquired ammonium much more efficiently than nitrate; even ammonium surge uptake can inhibit nitrate acquisition. Overall, this knowledge could provide the basis for optimizing culture practices such as initial biomass density and harvesting frequency, or even the fertilizer composition.

CRediT author contribution statement

Stephanie Revilla-Lovano: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.

Jose Miguel Sandoval-Gil: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing - original draft; Writing - review & editing.

José Antonio Zertuche-González: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.

María Dolores Belando-Torrentes: Data curation; Formal analysis.

Jaime Bernardeau-Esteller: Data curation; Formal analysis.

Laura Karina Rangel-Mendoza: Data curation; Formal analysis; Methodology.

Alejandra Ferreira-Arrieta: Data curation; Formal analysis; Methodology.

Jose Manuel Guzmán-Calderón: Data curation; Formal analysis; Methodology.

Raquel Muñiz-Salazar: Data curation; Formal analysis; Methodology.

Víctor F. Camacho-Ibar: Writing - review & editing.

María del Carmen Ávila-López: Data curation; Formal analysis; Methodology.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jose Miguel Sandoval Gil reports financial support was provided by Secretaría de Educación Píblica de México (NPTC project). Jose Miguel Sandoval Gil reports financial support was provided by Blue Evolution and Productos Marinos de las Californias (PROMAC).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102316.

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