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Effects of different culture media on growth, composition, quality and palatability of the green algae *Ulva* sp. cultivated in cylindrical photobioreactors

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ABSTRACT

Ulva spp. are valuable seaweeds with recognized commercial applications, including food, feed, and ecosystem services. Ensuring a sustainable and consistent supply of biomass with desirable profiles aligned with intended uses is fundamental for the successful applications of this seaweed. In this study, the growth rate, morphology, physiology, and composition of *Ulva* sp. produced by propagation in indoor cylindrical photobioreactors using four different culture media (lagoon water - LW, lagoon water enriched with Guillard medium (LF), with sea urchin wastewater - LU, and cow digestate - LD) was assessed; moreover, the nutrient uptake potential of the species was evaluated. The palatability and attractivity of the produced biomass towards the sea urchin *Paracentrotus lividus* were investigated. It was found that the media influenced all the parameters examined, the LF biomass weight was double compared to the other treatments and showed a slightly higher absorbance. Colorimetric analyses reported a significant darker color in *Ulva* sp. grown under enriched media. *Ulva* sp. showed higher nutrient removal potential in LF. The lipid content did not vary (2–3 % dry weight, DW), while the protein content ranged from 21 % in LF to 6–9 % in the other treatments. Carbohydrates and fiber content were significantly lower in LF (16 % and 30 %) compared to the other treatments, 27–34 %, and 41–48 %, respectively. Pigment content significantly varied, being higher in biomass grown in LF and LU. Sea urchins showed preferences for biomass grown under LU, followed by LD. This study shows how different nutrient sources affect the biochemical composition, growth, quality, and palatability of *Ulva* sp.. When cultivated under the synthetic enriched media (LF) the species exhibits characteristics better suitable for human consumption, although requiring a higher economic investment for production, while biomass derived from wastewater nutrients (LD, LU) confirms potential applications of the seaweed as valuable feed and for bioremediation services.

1. Introduction

The interest in seaweed aquaculture is growing worldwide accounting for one of the most productive mariculture crops, currently valued at US$15.3 billion [1]. Seaweeds are extensively produced in Asian countries, although to date practices and research are increasing also in Western countries with numerous projects and economic investments [2–5]. Western seaweed aquaculture currently contributes 0.8 % to global production [6]. This contribution is attributed to a handful of species (e.g. *Laminaria japonica*, *Eucheuma* spp., *Gracilaria* spp., *Undaria pinnatifida*, *Porphyra* spp., *Kappaphycus alvarezii*, *Sargassum fusiforme*) that are exploited for their commercial applications, such as food, feed, fertilizer, biofilter, and biomaterial [1].

Among green algae, the species belonging to the genus *Ulva* (Ulvophyceae, Chlorophyta), also known as sea lettuce, are the most common, widely distributed, and one of the most exploited genera [7]. These algae can adapt to various habitats and environmental factors (e.g. light, nutrients, temperatures, etc.) and can grow reproductively,

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parthenocarply and asexually via biflagellate zoospores [8], which makes it an ideal candidate for laboratory experiments. *Ulva* spp. are valuable seaweeds with numerous recognized commercial applications, including fertilizer, nutraceutical, biomaterial, biofilters applications, human (e.g. "aonori"), and animal feed (e.g., abalone, shrimps, and sea urchin) [9–11]. The biochemical composition of *Ulva* spp. has been extensively analyzed showing that it is rich in proteins, dietary fibers, minerals, vitamins, and bioactive secondary metabolites [7], indicating that it is suitable for human consumption and has applications in the feed industry. Moreover, sea lettuce is considered a strong attractant and feeding stimulant, able to increase the feeding intake and foster the conversion rate of marine herbivores [12]. For example, it is largely studied concerning the feeding and the feed formulation for the valuable sea urchins *Paracentrotus lividus* [13–16]. The biochemistry of the species can be highly affected by numerous factors such as season, growth stage and environmental factors among which the most important are temperature, nutrient availability and light irradiation [17–24]. For example, elevated temperature and irradiance led to a reduction of protein content, whereas the introduction of dissolved inorganic nitrogen boosted protein and fatty acid levels and reduced ash content [22]. On the other hand, this large variability in *Ulva* spp. composition is a challenge for the commercialization, especially when ensuring precise quality is crucial for its successful exploitation [25].

Various studies have been conducted investigating laboratory and in-field cultivation (in tank or sea-based) of various species of *Ulva* spp., either by sexual reproduction or by propagation [7]. More recently, progress has been made in growing *Ulva* spp. in photobioreactor systems besides open ponds and sea farming, which can range from laboratory to industrial scale systems and are raising interest due to the controlled environment where conditions such as light, nutrients, temperature, and CO_2, can be closely regulated. Photobioreactors already tested for *Ulva* spp. production range from panel to tubular systems, with indoor or outdoor setups [26–28]. These systems are more expensive and sometimes with a higher footprint than coastal and offshore cultivation; however, they are flexible and can provide tailored biomass with sought-after composition, resulting in higher value, which might justify the costs of setting up and running photobioreactor systems [29].

Another important and recognized use of *Ulva* spp. is as biofilter and bio-remediator with the ability to absorb and sequester nutrients such as nitrogen, phosphorous, and carbon. An important application is the bioremediation of nutrient-rich wastewater from intensive land-based aquaculture [30,31], and carbon sequestration [32]. Moreover, biomass of *Ulva* spp. has shown to be able to accumulate and reduce the concentration of metals, nitrogen, and phosphorus that might be derived from agricultural activities, invertebrates or finfish effluents, making it an ideal biofilter [7].

Previous research on *Ulva* sp. used in integrated systems with other species such as sea bream, abalone, and sea urchins [14,31,33] at laboratory and commercial scale (Wild Cost Abalone, South Africa [34]), showed the potential for assimilation of inorganic nutrients, optimizing the need for resources, and decreasing the ecological impact of wastewater [35], often resulting in biomass with higher quality (e.g. increment in protein content) [36]. This enriched biomass can be further processed and biorefined for downstream applications (e.g. polysaccharides and amino acids extractions, biomaterials, biofuel) and to increase the profitability of aquaculture activities [36]. Ensuring a sustainable and consistent supply of biomass with targeted and desirable biochemical profiles aligned with intended uses is fundamental for the successful applications of this species [7]. Furthermore, it is important to understand the relationships and/or influences of different nutrients from various effluents on the algal physiology, biomass composition, and morphology.

Hence, this study investigates 1) the cultivation by propagation of *Ulva* sp. in indoor cylindrical photobioreactors, considering different media as sources of nutrients (Nitrogen and Phosphorus) and their effect on the growth, morphology, physiology, biochemical composition, nutrient uptake efficiency, and overall quality of the biomass produced, 2) the influences of the biochemical composition of *Ulva* sp. on its palatability and attractivity towards the model species *P. lividus*.

2. Material and methods

2.1. Samples collection and pre-treatment

Wild biomass and lagoon water were collected in June 2023 from a coastal lagoon of South Sardinia, Italy (Santa Gilla, Lat 39°13′50.67"N, Long 9° 4'49.34"E) and transferred to the laboratory. This lagoon is one of the most productive sites for commercial production of bivalves; it is subjected to frequent water quality assessments and classified as "B" (i.e. site for collection of bivalves marketed for human consumption after purification, [37]).

The lagoon water used for the experiment was treated with 60-μm sand filter, protein skimmer, ozone treatment, a set of filters (50 μm, 10 μm, 1 μm), activated carbon, and UV sterilization system.

The collected seaweed was acclimated in indoor tanks with filtered lagoon water at room temperature (between 20 and 25 °C) and a natural light cycle for one week before the trials. Morphological analyses and previous observations of the species in the lagoon [38], allowed us to identify the species as *Ulva* sp. with foliose morphology. Before the biochemical analyses, the samples were freeze-dried for 48 h.

2.2. Experimental setup

On the first day of the experiment, the biomass was rinsed with fresh water to remove attached organisms and biofouling. The central sections of the blades were cut with scalpels into discs of 8–10 cm to ensure homogeneity of the cultured biomass and blotted dry with a paper towel to remove the excess water. About 20 g (± 0.5) of fresh weight biomass was placed per bioreactor filled at 20 L level, resulting in a final density of 1 g L^{-1}. Twelve photobioreactors (PBR, plexiglass cylinders, diameter 16 cm, tall 180 cm, thickness 2.7 cm) were located in a controlled temperature room set at 20 °C with aeration provided by air stones from the bottom and under artificial LED lights (two LEDs each PBR, Futura 125 cm, Natural Indoor spectrum) (Fig. 1). Light intensity for each bioreactor was measured at 4 points (front, back, right, and left of the cylinder) using a light meter (Li-Cor Inc., Lincoln, NE, USA) with an overall reported average of 83 μmol m^{-2} s^{-1} ± 10.4 SD.

Lagoon water was used as baseline and stock solution to prepare other culture media. Four types of nutrient media were tested in batch photobioreactors and compared in triplicate ($n = 12$): 3 PBR contained lagoon water (LW), 3 PBR lagoon water enriched with F/2 (LF) [39], (see supplementary Table 1 for F/2 medium composition), 3 PBR lagoon water enriched with sea urchin wastewater (LU) collected from the Experimental Aquaculture laboratory for sea urchins production in Santa Gilla (grow-out units, for more details, see Pani et al. [40]), and 3 PBR lagoon water enriched with digestate (LD). Digestate used in the experiment was collected at a mesophilic anaerobic digestion plant treating livestock effluent and grass silage, located in southern Sardinia (Arborea, Italy) and stored at 4 °C. The digestate was characterized according to Attene et al. [41]. The LD medium was prepared by adding 1 mL L^{-1} of digestate to the lagoon water.

The growth experiment lasted one week, during which water temperature was measured using HOBO loggers and kept at 23.3 °C ± 0.5 SD, whereas salinity was measured with a multiparameter probe (In-Situ SmarTROLL Multiparameter Handheld) and kept at 32.5 PSU ± 0.7 SD.

2.3. Nutrient uptake and removal

The dissolved nitrogen (nitrite, nitrate, and ammonia, expressed in mg L^{-1}) and dissolved phosphorus content (total phosphorus, phosphate, and phosphorus pentoxide, expressed in μg L^{-1}) in LW and in prepared media (LF, LU, LD, Table 1) were analyzed in triplicate both at

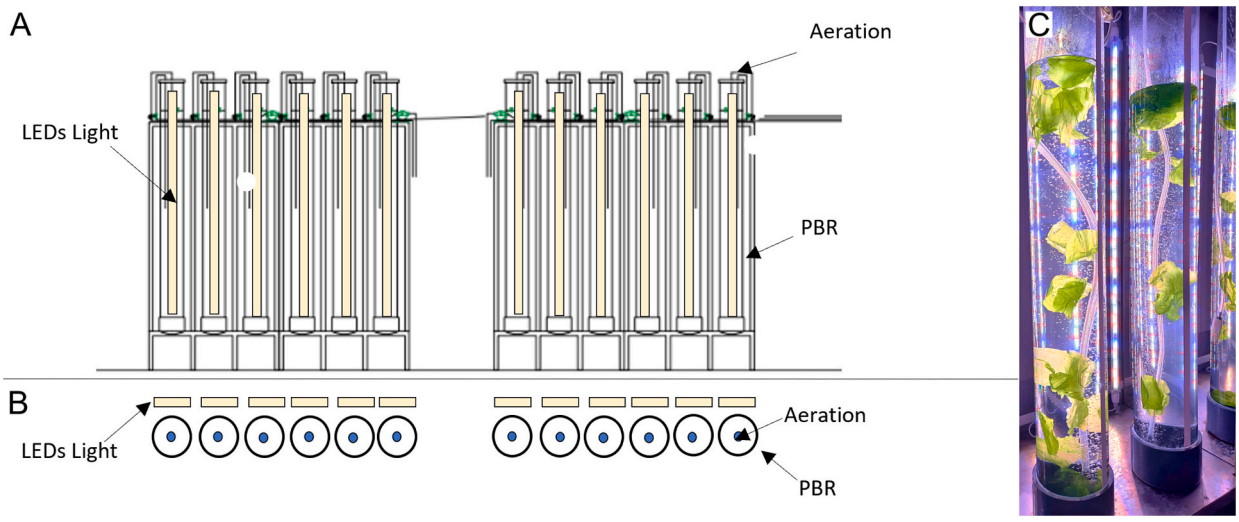

Fig. 1. Schematic from A) front and B) above of the 12 photobioreactors used in the experiment). C) picture of PBRs. The LED lights are placed vertically behind each photobioreactor (PBR), covering the full length and the air is provided from the top through an airline that terminates with a weighed air stone at the bottom of the PBR.

Table 1
Nutrient concentration at T0 and T*final* in the four different trials (LD (lagoon water enriched with digestate), LF (lagoon water enriched with F/2), LW (lagoon water), LU (lagoon water enriched with sea urchins wastewater). Data are reported as average ± SE.

Culture media	Phosphorus (μg L^{-1})					
	P-Tot		PO_4^{3}		P_2O_5	
	T0	T*final*	T0	T*final*	T0	T*final*
LD	166 ± 47.27	20.33 ± 4.84	509.33 ± 145.32	62 ± 14.53	380.67 ± 108.46	46.33 ± 10.84
LF	868.67 ± 303.93	32 ± 10.44	2664.67 ± 931.07	92.33 ± 32.74	1990.67 ± 695.37	78.33 ± 24.33
LW	113 ± 10.58	8.33 ± 0.67	346.67 ± 32.57	24.67 ± 1.33	259 ± 24.38	19 ± 1.00
LU	543 ± 41.02	21 ± 2.65	1668 ± 126.05	64.33 ± 8.01	1246.33 ± 94.36	47.67 ± 6.01

Culture media	Nitrate (mg L^{-1})		Nitrite (mg L^{-1})		Ammonia (mg L^{-1})	
	T0	T*final*	T0	T*final*	T0	T*final*
LD	0.53 ± 0.04	0.37 ± 0.01	0.25 ± 0.02	0.04 ± 0.02	1.88 ± 0.52	0.75 ± 0.15
LF	6.87 ± 2.94	0.35 ± 0.12	0.19 ± 0.01	0.91 ± 0.49	1.32 ± 0.49	1.11 ± 0.24
LW	0.3 ± 0.07	0.23 ± 0.08	0.19 ± 0.02	0.23 ± 0.01	0.76 ± 0.32	0.92 ± 0.19
LU	2.43 ± 0.12	0.17 ± 0.03	0.24 ± 0.01	0.21 ± 0.01	0.82 ± 0.16	1.15 ± 0.14

the beginning (T0) and at the end of the experiment (T *final*).

The samples were analyzed using a spectrophotometer set at 525 nm for nitrate, 480 nm for nitrite, 425 nm for ammonia, and 610 nm for phosphorus (HI-801-02 iris, HANNA Instruments®) and respective reagent kits for each component in seawater (HANNA Instruments®; HI764 marine nitrite low range NO_2-N; HI782 marine nitrate high range NO_3^-N; HI3826 marine ammonia low range, NH_4; HI736–25 phosphorus marine ultra-low range, P-tot). The sum of NO_3^-N; NO_2-N and NH_4 was used to estimate the dissolved inorganic nitrogen (DIN). The ratio between DIN and Ptot (N/P) was calculated at T0 and T*final* in all culture media.

The reduction in nutrient concentration between the time intervals (T0 and T*final*) is expressed as a percentage and defined as nutrient uptake efficiency (NUE) and was calculated according to Massocato et al. [36] assessing the changes in nitrogen (as nitrate and ammonia) and total phosphorus concentrations:

$$\text{NUE}\ (\%) = 100 - \frac{(Ctfinal \times 100)}{Ct0} \quad (1)$$

where Ct0 represents the initial concentration of nutrients and C*tfinal* represents the concentration of nutrients at the end of the experiment.

The amount of nutrients removed per unit of time per volume by seaweed wet weight represents the nutrient uptake rate (NUR) and is determined from changes in ammonia, nitrate, nitrite, DIN and total phosphorus (P-tot) according to Duan et al. [42]:

$$\text{NUR} = \frac{[(\text{Ct0} - \text{Ctfinal}) \times \text{V}]}{\text{WW} \times \text{t}}$$

where Ct0 represents the initial concentration of nutrients, C*tfinal* represents the concentration of nutrients at the end of the experiment, V is the PBR volume (L), WW is the fresh weight (g) at T0 and t is the duration of the experiment (h). Results are expressed as μmol g^{-1} WW h^{-1} (WW is the wet weight of the biomass).

2.4. Growth assessment

At the end of the experiment, the biomass was blotted dry and weighed with an analytical balance (± 0.001 g) to calculate the specific growth rate (SGR), expressed as a percentage per day and calculated according to Mantri et al. [7]

$$\text{SGR} = \frac{[ln\,(\text{Wf}) - ln(\text{W0})]}{\text{t}} \times 100$$

where Wf was the final fresh weight after t days of culture (7 in this study), and W0 was the initial fresh weight.

2.5. Morphology and physiology

The colorimetric analyses of *Ulva* sp. were conducted for each treatment on the algal thallus in triplicates for subsamples using a digital colorimeter (Chroma meter CR-400, Konica Minolta, Tokyo, Japan), considering three parameters, L* = lightness, a* = red/green and b* = yellow/blue (CIELAB) [43,44]. The colorimeter data at T0 are reported in Table 2.

The absorbance of the algal thallus (on 12 randomly selected blades at T0 and three blades for each treatment at T*final*) was determined using a quantum/radiometer/photometer (LI – 1500, Light Sensor Logger, Li-Cor Inc., Lincoln, NE, USA) [45]. The algal thallus was placed on top of a microscope slide (previously read as blank) illuminated with a stereo microscope with a luminescence source (See supplementary Fig. 1). Absorbance (A) was calculated according to Beer–Lambert law:

$$A = 2 - log_{10}\ T\%$$

where T (transmittance) is the ratio between transmitted irradiance (Et) and the incident irradiance (Eo) of the microscope lamp expressed as percentage, determined by the Li-Cor sensor. The absorbance at T0 was 0.375 ± 0.024SD.

2.6. Biochemical composition

At the end of the trials, the biochemical composition of *Ulva* sp. was determined on samples from different culture media. Samples of algal biomass were frozen at −80 °C and subjected to freeze-drying (Model LIO5P-Digital, 5 Pascal, Trezzano sul Naviglio, Milan, Italy). The determinations included the proximate composition (residual moisture, ash, total carbohydrates, Neutral Detergent Fiber (NDF), total lipid, and protein), polyphenols, and pigments (chlorophyll-a, chlorophyll-b, and carotenoids).

2.6.1. Chemicals

Methanol (MeOH), hydrochloric acid (HCl), sodium hydroxide (NaOH), and chloroform ($CHCl_3$) were ultra-residue solvents of analytical grade purchased from Merck (Darmstadt, Germany). Sulfuric acid (H_2SO_4) (96 %) and (0.5 N), sodium hydroxide (NaOH) (32 %, 0.5 N and 1 N), Folin-Ciocalteu reagent, phenol, KCl, Na_2CO_3, Na_2SO_4 anhydrous, $CuSO_4$, PBS pH 7.4, $Na_2B_4O_7xH_2O$, EDTA, $C_{12}H_{25}NaO_4S$, triethylene glycol, Na_2HPO_4, D-glucose, Gallic acid, and α-amylase were reagent grade purchased from Sigma Aldrich (Chemie, Munich, Germany). Double-deionized water with a conductivity of <18.2 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

Table 2
Results of the colorimetric analysis of the biomass at the beginning of the experiment (t0) and under the different culture media LW (lagoon water); LD (lagoon water enriched with digestate), LF (lagoon water enriched with F/2), LU (lagoon water enriched with sea urchins wastewater). The colors were defined using the CIELAB coordinators converted with the online tool ColorHexa (https://www.colorhexa.com/) and reported in the column Color. Data are reported as average ± standard error. L* = lightness, a* = red/green and b* = yellow/blue.

Treatment	L*	a*	b*	Color
t0	63.74 ± 2.97	−24.39 ± 0.27	62.43 ± 1.82	
LW	72.13 ± 0.66	−17.49 ± 0.83	51.3 ± 2.09	
LD	67.13 ± 1.12	−20.66 ± 1.64	58.25 ± 0.63	
LF	55.75 ± 1.46	−25.76 ± 0.55	58.1 ± 1.70	
LU	65.56 ± 0.80	−21.98 ± 1.48	60.2 ± 0.51	

2.6.2. Moisture and ash

The freeze-drying process left an average of 3.4 % residual moisture; before carbonization, the samples were oven-dried at 105 °C for 24 h. Subsequently, 0.5 g of samples were carbonized at 525 °C in a porcelain crucible for 8 h for total ash analysis. Ash is expressed as $g100g^{-1}$ freeze-dry weight (DW).

2.6.3. Total carbohydrates

Carbohydrates analysis was performed by the phenol-sulfuric acid method, according to Dubois et al. [46]. Briefly, 20 mg of freeze-dried sample were placed in a 15 mL falcon tube with 5 mL of HCl 1 M, sonicated in a bath for 15 min, and extracted in a boiling-water bath (100 °C) for 1 h. A hundred microliters of the obtained extract were diluted with double-deionized water to a final volume of 1 mL, then mixed in a glass tube with 1 mL of a 5 % (*w/v*) phenol solution in deionized water and 5 mL of H_2SO_4 (95 %). The solution was gently mixed, left to stand at room temperature for 30 min, and finally read at 488 nm using a UV–Vis spectrometer Cary 50 (Varian Inc., Palo Alto, CA, USA). Total carbohydrates are expressed as $g \times 100\ g^{-1}$ DW of equivalent in D-glucose. For the quantification, a 5-point calibration curve of D-glucose (20–100 mg L^{-1}) was prepared and considered acceptable with $r^2 \geq 0.997$.

2.6.4. Total lipids

Total lipids quantification was carried out according to Chen et al. [47]. Ten milligrams of freeze-dried sample were suspended in 40 μL of PBS 0.05 M (pH 7.4) plus 460 μL of a NaOH (1 N)/MeOH solution (3:1) and processed in the vortex for 10 min in the presence of glass beads. The obtained suspension was diluted with 1 mL of NaOH (1 N)/MeOH (75/25), vortexed again for 5 min, and heated (100 °C for 30 min) to allow saponification. The suspension was cooled down to room temperature and centrifuged at 3154 ×*g* and 10 °C for 5 min to precipitate cell debris. One milliliter of the supernatant was transferred to a 15 mL falcon tube, mixed with 3 mL of $CHCl_3$/MeOH (2/1) plus 0.5 mL of a KCl solution 0.88 % (*w/v*), vortexed, and centrifuged at 3154 ×*g*, for 10 min. One milliliter of the organic phase was transferred to a 2 mL HPLC vial and evaporated under a gentle nitrogen stream. Finally, the remaining fat residue was weighed. Total lipids are expressed as $g \times 100\ g^{-1}$ DW.

2.6.5. Proteins

Protein content was evaluated according to the Kjeldahl method [48]. Briefly, 0.5 g of freeze-dried sample was placed in a Kjeldahl flask, followed by the addition of 0.5 g of Na_2SO_4, 10 mg of copper sulfate ($CuSO_4$), and 20 mL of H_2SO_4 96 %. The samples were placed in a Speed-Digester K-436 BÜCHI (Labortechnik GmbH, Essen, Deutschland) and submitted to a digestion process at 400 °C until the solution became colorless. The Kjeldahl flasks were then removed, allowed to cool, and introduced into a distillation system VAPODEST 300 (C. Gerhardt GmbH & Co. KG, Königswinter GERMANY). A hundred milliliters of Milli-Q water and 80 mL of NaOH 32 % (*w/v*) were added to the Kjeldahl flask automatically. In the 250 mL receiving flask, 10 mL of H_2SO_4 0.5 N and 10 drops of methyl red indicator were manually added. After 4 min of distillation, the receiving flask was removed and titrated with NaOH 0.5 N. The reaction was considered complete when the solution changed color from red to light yellow. The total protein content expressed as $g \times 100\ g^{-1}$ DW was calculated with the following formula:

$$\%protein = \frac{(a-b) \times c \times 100 \times K}{g}$$

where "a" mL of H_2SO_4 0.5 N added to the collection flask (10 mL); "b" mL of titrant used (NaOH 0.5 N); "c" is the conversion factor mL of

H_2SO_4 0.5 N in g of nitrogen (0.007); "K" is the general nitrogen–protein conversion factor (6.25), and "g" grams of sample.

2.6.6. Neutral detergent fiber (NDF)

The determination of Neutral detergent fiber (NDF) was performed according to Van Soest et al. [49] using a FIWE Advance Automatic Fiber Analyzer, VELP Scientifica, (Usmate Velate (MB), Italy). Briefly, 0.5 g of freeze-dried sample and 0.5 g of Na_2SO_3 were placed in a P2 glass crucible and subjected to a 1.5-h digestion process in neutral surfactant mixture (6.81 g $Na_2B_4O_7xH_2O$ + 18.61 g EDTA +30 g $C_{12}H_{25}NaO_4S$ + 10 mL triethylene glycol +4.56 g Na_2HPO_4 in 1000 mL of double-deionized water), plus α-amylase. The obtained samples were dried in an oven for 8 h (100 °C) and carbonized for 5 h at 525 °C. The concentration of NDF is expressed as g × 100 g^{-1} DW and calculated with the following formula:

$$\text{NDF}\ (\%) = \frac{(W1 - W0)}{g} \times 100$$

where "W1" indicates the crucible weight (g) + sample weight after drying, "W_0" is the crucible weight (g) + sample weight after carbonization, and "g" is the grams of sample (0.5 g).

2.6.7. Chlorophylls (a and b) and total carotenoids

The concentration of chlorophylls (a and b) and total carotenoids was determined according to Singh and Singh [50]. Twenty milligrams of freeze-dried sample were weighed in a 15 mL falcon tube plus 4 mL of MeOH, vortexed for 2 min, and left in a thermostatic bath at 70 °C for 3 min. After cooling, the tubes were centrifuged at 3154 ×*g*, 10 °C for 5 min to precipitate the undissolved cell debris. The bright green supernatant was analyzed in a UV–Vis spectrometer Cary 50 (Varian Inc., Palo Alto, CA, USA). The wavelengths analyzed were 470 for carotenoids, 653, 666 for chlorophyll *a* and b, and 750 nm for the impurities. The concentration of chlorophylls (a and b) and total carotenoids is expressed by using the following formulas [51]:

$$\text{Chl a mg L}^{-1} = 15.65\ (A666 - A750) - 7.34\ (A653 - A750)$$

$$\text{Chl b mg L}^{-1} = 27.05\ (A653 - A750) - 11.21\ (A666 - A750)$$

$$\text{Carot mg L}^{-1} = \frac{1000(A470 - A750) - 2.860\ (\text{Chl} - a) - 129.2\ (\text{Chl} - b)}{245}$$

The final concentration of chlorophylls (a and b) and total carotenoids was expressed in mg kg^{-1} DW. Chlorophylls (a and b) and total carotenoids concentration obtained with the reported formulas (mg L^{-1}) were multiplied by the extraction volume expressed in L and divided by the sample weight expressed in kg.

2.6.8. Total polyphenols determination

The determination of the total polyphenolic content was performed using the Folin–Ciocalteu reagent method [52]. 0.5 g of freeze-dried samples were placed in a 15 mL falcon tube plus 5 mL of a MEOH/H_2O mixture (80:20 *v*/v). The tubes were shaken for 1 min in a vortex and for 15 min in a rotary shaker. Finally, the samples were centrifuged at 3154 *g* and 10 °C for 15 min. 100 μL of the extract solution was put in a 10 mL calibrated flask with 500 μL of Folin-Ciocalteu reagent, 1 mL of a sodium carbonate solution 20 % (p/v), and MilliQ water till 10 mL. The mixture was agitated for 1 min in a vortex and incubated for 80 min at room temperature in the dark. Quantitative analyses were carried out with a UV–Vis spectrometer Cary 50 (Varian Inc., Palo Alto, CA, USA) set at 750 nm. For the quantification, a 5-point calibration curve of Gallic acid (50–500 mg× L^{-1}) was prepared and considered acceptable with $r^2 \geq 0.997$. Total polyphenols are expressed as mg× kg^{-1} DW.

2.6.9. Palatability preference trial with sea urchin (Paracentrotus lividus)

The cultivated biomass was used in a multiple-choice experiment to carry out a preliminary assessment of the diet preference of sea urchins *P. lividus* following an already established methodology by Addis et al. [12]. Although this is a small-scale study, it can provide valuable preliminary insights. The experiment was carefully designed to minimize variability and maximize the reliability of the results. The sea urchins were exposed to algae grown under four different media in a controlled environment, after starvation, and measuring indicative parameters, such as speed to the target and tortuosity that help to identify and isolate the effects of the media on diet preference. In detail, six sea urchins ($n = 6$) with a diameter comprised between 2 and 3.5 cm were randomly collected from the batch of juveniles produced in the hatchery side of the Experimental Aquaculture laboratory of the University of Cagliari (Italy) and cultured according to the procedure described by Carboni et al. [53] and Hannon et al. [54]. The sea urchins were starved for 48 h before the trial. Each sea urchin was allowed to explore the experimental arena, consisting of a circular plastic tank (30 cm in diameter, 8 cm high) containing 4 L of seawater, that was provided with a choice of four diets consisting of *Ulva* sp. biomass grown under different culture media (LF, LW, LU, LD). The four diets were randomly disposed at the four poles of the experimental arena to randomize the position of the diet in the arenas. To prevent algal floating, each diet (about 500 mg) was inserted into a ceramic filter ring. At the beginning of the experiment, the sea urchins were placed in the center of the arena (Supplementary Fig. 2). The trials lasted 15 h and were video-recorded for subsequent behavioral analysis by using a Samsung SMX-F34 (Samsung, Seoul, Korea) color digital camera mounted above the test tank.

The behavior was explored using a combination of manual coding methods (to define the first and second choice, the time to the target, the tortuosity to the target, and the speed) and the ANY-maze tracking software (Wood Dale, IL, USA), to produce the track plot and the heat map and calculate the speed, tortuosity and time to first and second choice of the sea urchins.

2.7. Statistical analyses

Data were assessed for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test >0.05) prior to ANOVA analyses and transformed when necessary. One-way ANOVA (at 95 % confidence interval) was performed for SGR, biochemical composition, absorbance, and colorimeter data, with media (4 levels, LW, LF, LU, LD) as a factor. One-way ANOVA with Time as a factor (2 levels, T0 and Tfinal) was performed on nutrient concentrations. Significant differences were tested with Tukey's post-hoc test (Sigma Plot 14.0).

Differences in nutrient uptake (NUE and NUR) were assessed with permutational analyses of variance data (PERMANOVA) in univariate contexts with Media (4 levels, LW, LF, LU, LD) as a factor. Multivariate analyses were performed on biochemical composition and color, including non-metric multidimensional scaling (NMDS) [55] based on Euclidean distances (with accepted stress values <0.20 and ideally <0.10 according to Clarke, [56]. Monte Carlo PERMANOVA (v. 1.0.5) test with 999 permutations was performed, followed by pairwise test when significant differences were identified and combined with a test for homogeneity of dispersions within factor groups performed using PERMDISP [57] with distance to centroids (PRIMER 7). PCA analyses on normalized data of the overall biochemical composition of the biomass under the four treatments were performed, accounting for % variation and eigenvectors, and represented with PCA loading plot (PRIMER 7). Correlation analyses using Pearson correlation with $P < 0.05$ were applied to investigate relationships between the analyzed parameters (Sigma Plot 14.0).

The results of the palatability preference trial were analyzed using χ^2 to test the null hypothesis that the frequency observed in the first choice were not dependent on the type of *Ulva* considered (LW, LU, LD, LF). We compared the number of individuals selecting each *Ulva* type expressed as percentage on the total number of sea urchin tested.

3. Results

3.1. Growth assessment

The SGR was the highest for the biomass cultivated in LF, which was more than double (9.86 ± 0.52 % day^{-1}, mean ± SE) of the cultures grown in LD (4.53 ± 1.29) and LU (4.25 ± 1.8) and the lowest was observed in LW (3.74 ± 0.49). The statistical analyses showed significant differences in SGR among treatments ($F_{(3,8)} = 16.47$, $P < 0.001$), with the exception of the comparison of LD, LW, and LU (Fig. 2).

3.2. Morphology and physiology

The absorbance of the biomass grown with LF was slightly higher compared to all other treatments, followed by LU, LD, and finally LW (Fig. 3) without significant differences ($F_{(3,8)} = 3.33$, $P = 0.077$) among the treatments at T*final*.

The colorimetric analyses CIELab showed significant differences for all three parameters at T*final* (One-way ANOVA, a*, $F_{(3,8)} = 7.99$, $P = 0.009$, b* $F_{(3,8)} = 7.68$, $P = 0.010$, L*, $F_{(3,8)} = 41.97$, $P < 0.001$), where L* and b* for LW were statistically different from the others. Moreover, LW also significantly differed from LF for a* values, corresponding to a paler biomass for LW cultures and to a darker green color for the biomass grown under F2 (Table 2).

A negative correlation was reported (Pearson correlation) between L* and the absorbance ($r = -0.757$, $P = 0.004$), a positive correlation between b* and the absorbance ($r = 0.593$, $P = 0.04$), and no correlation between a* and absorbance ($P > 0.05$). A significant negative correlation was reported between a* and L* and the protein content ($r = -0.77$ and $r = -0.92$, respectively), indicating that "greener" and darker biomass corresponded to higher protein content (Supplementary Fig. 3, Table 3).

3.3. Nutrient uptake

A considerable decrease was observed in phosphorus concentration (One way ANOVA, T0 vs T*final*) among culture media at the end of the experiment. The media LF and LU showed the highest decrease accounting at *Tfinal* for 4 % of the initial value, whereas LW and LD had the lower decrease with values of 7.3 and 12.2 %, respectively. Ammonia levels did not vary over time, while nitrate levels showed a 20-fold decrease in the LF treatment from T0 to T*final*. Conversely, nitrite was

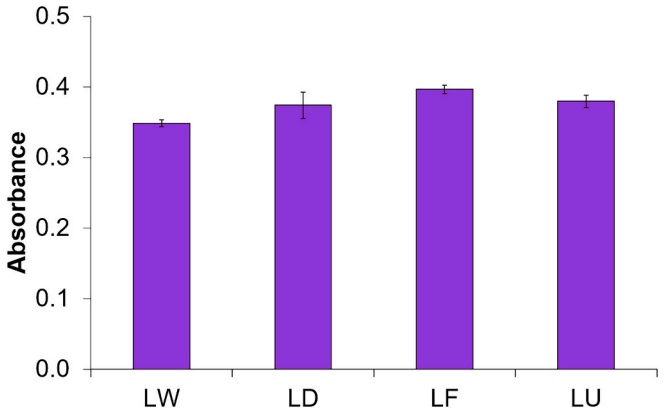

Fig. 3. Absorbance, at T *final* of cultures grown under different media: LW (lagoon water); LD (lagoon water enriched with digestate), LF (lagoon water enriched with F/2), LU (lagoon water enriched with sea urchinswastewater); measured with a Lycor® light meter under a transmitted light microscope. Error bars indicate standard error ($n = 3$).

Table 3
Correlation results, including r and *P* values from Pearson analyses for absorbance, colorimeter data (a* = red/green, b* = yellow/blue and L* = lightness), pigment (Chlorophyll a (Chl-a), Chlorophyll b (Chl-b), carotenoid) and protein content. In bold are highlighted significant values.

Variables	ABS (%)		Protein (% DW)	
	r	P value	r	P value
a*	−0.569	0.053	−0.765	**0.003**
b*	0.593	**0.04**	0.316	0.317
L*	−0.757	**0.004**	−0.924	**<0.001**
Chl-a	0.693	**0.012**	0.936	**<0.001**
Chl-b	0.662	**0.019**	0.917	**<0.001**
Carotenoids	0.694	**0.012**	0.490	0.106

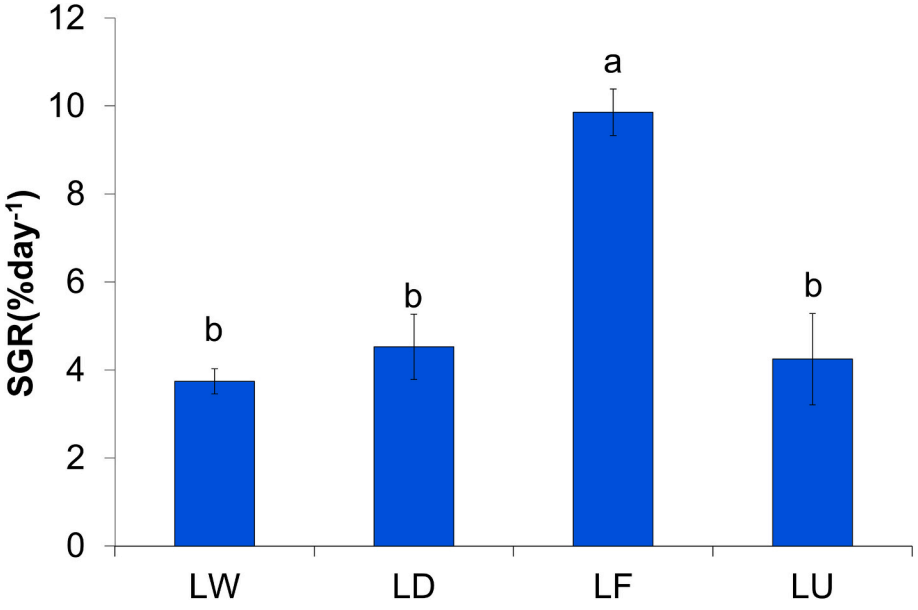

Fig. 2. SGR (% day^{-1}) of cultures grown under four different culture media, LW (lagoon water); LD (lagoon water enriched with digestate), LF (lagoon water enriched with F/2), LU (lagoon water enriched with sea urchins wastewater). Letters above bars indicate significant differences (Tukey's post-hoc test). Error bars indicate standard error ($n = 3$).

nearly 5 times higher at T*final* in LF, with no observable differences during the experiment in the other treatments (Table 1). The N/P ratio at T0 was maximum in LD (11.0), followed by LW (7.5), LF (3.2) and minimum in LU (2.6), whereas in T*final* it was maximum in LW (119.6), followed by LF (58.0), LU (54.3), and minimum in LD (34.6).

Ulva sp. showed different removal capacities of nitrogen and phosphorus, according to the media tested. The NUE for DIN was statistically higher for seaweed produced with LD (2 to 3 times higher), followed by LF and LU, whereas reported negative values for LW. Nitrate uptake was higher (ca. 3 times) in LF and LU and lower in LW and LD, and equivalent removal rates were reported among media for uptake of ammonia and nitrite (Table 4). A significant difference was reported in NUE for Ptot, where LD was lower than LF and LU (Table 4). Overall, the NUR rate was higher for media with higher sources of nutrients, showing higher efficiency for nitrate, followed by ammonia and nitrite (Table 3). Significant differences were observed for nitrate and Ptot with greater values observed in LD and LF, followed by LU, whereas negative values were observed in LW. On the other hand, differences for DIN ammonia and nitrite were not significant (Table 4).

3.4. Biochemical analyses

The biochemical analyses showed that the lipid content in *Ulva* sp. did not vary among treatments (2–3 % DW, $P > 0.05$). The protein levels exhibited significant variation among treatments ($F_{(3,8)} = 165.29$, $P < 0.001$). The proteins in LF treatment showed the highest values (21 % DW), followed by all other treatments (9–6 % DW). Conversely, carbohydrates and fiber content were lower in LF (16 % and 30 % DW, respectively) compared to other culture media (27–34 % and 41–48 % DW, respectively). Residual moisture and ash contents did not vary among treatments ($P > 0.05$, Fig. 4).

Polyphenol contents did not vary (Fig. 5A), while Chl-a and b varied among culture media ($F_{(3,8)} = 24.75$, $P < 0.001$, $F_{(3,8)} = 18.36$, $P < 0.001$, respectively). The highest values were reported in biomass grown in LF, followed by LU, LD, and LW (Fig. 5B and C). Carotenoid concentrations (as mg kg^{-1}) varied among treatments, being double in concentration in LF (67.53 ± 4.5 SE) and LU (65.44 ± 7.63 SE) compared to LW (32.51 ± 3.81 SE) ($F_{(3,8)} = 5.61$, $P = 0.023$), while LD reported 46.53 ± 5.76 SE, which was not significantly different ($P > 0.05$) (Fig. 5D).

PCA analyses carried out considering the overall biochemical composition (proximate, pigments, and polyphenols) showed that 75.9 % of the variance was explained by PC1 and PC2 (% cum.). PC1 (60 %) had a large positive association with protein, lipid, ash, Chl-a, and Chl-b. PC2 (15.9 %) had a large negative association with lipids and polyphenols. PC3 (14.2 % variation) had a strong negative association with polyphenols and carotenoids (Supplementary Table 2). LF biomass is clearly separated from the other treatments, mainly characterized by a higher content of protein and chlorophyll. Biomass grown under wastewater (LD and LU) showed a more homogenous distribution characterized by higher carbohydrates, fiber, carotenoids, and polyphenols content (Fig. 6).

A positive correlation was reported between absorbance and Chl-a, Chl-b, and carotenoids ($r = 0.693$ and 0.662, $r = 0.694$, respectively). A positive correlation was observed also between protein and Chl-a and Chl-b (Table 2). Correlation analyses (Pearson correlation) showed a strong negative relationship between a* and the Chl-a ($r = -0.75$, $P < 0.001$), Chl-b ($r = -0.67$, $P = 0.02$), and carotenoids ($r = -0.66$, $P = 0.02$). A strong negative correlation was reported also between L* and Chl-a ($r = -0.90$, $P < 0.001$), Chl-b ($r = -0.89$, $P < 0.001$), and carotenoids ($r = -0.65$, $P = 0.02$) (Supplementary Fig. 4 and 5).

The multivariate PERMANOVA analyses considering the biochemical composition (proximate, polyphenols, and pigments) and the color parameters (L*, a*, and b*) reported significant differences in composition and morphology among the culture media (Pseudo $F_{(3,8)} = 5.93$, P (MC) = 0.001), except between LD and LU (Pairwise, $t = 1.04$, P(perm) = 0.38) and LD and LW ($t = 1.37$, P(perm) = 0.16). These differences were not dependent on dispersion of the groups (PERMDISP, P-perm = 0.235) (Supplementary Fig. 3).

3.5. Palatability preference trial with sea urchin (*Paracentrotus lividus*)

Of the 6 sea urchins used for the trial, 3 of them selected biomass grown in LU as the first choice of diet, 2 selected the biomass grown in LD, and one biomass grown in LW. The χ^2 test revealed a significant difference in the frequency of choice selection, with LU (50 %) and LD (33.33 %) significantly more selected than the others (LW = 16.67 %, LF = 0 %) (Supplementary Table 3A). The first choice happened between 22 and 27 min from the start of the experiment and the tortuosity of the first choice was included between 1 and 1.27, showing an almost linear trajectory towards the first target. The speed at which the sea urchins traveled towards the first choice was included between 0.43 and 0.61 cm/min (Supplementary Table 3B, Fig. 7). Four sea urchins also selected a second choice, where two touched the biomass grown in LF, one in LW, and one in LD.

4. Discussion

The use of cultivated seaweed for both animal feed and human consumption is increasingly growing [58]. There is a significant interest in seaweeds produced with different culture media possibly reusing waste materials as a source of nutrients in the perspective of the circular economy and the potential contributions of seaweeds to multiple Sustainable Development Goals (SDGs) [59].

Among the candidate species, sea lettuce is one of the most suitable for human consumption, feeding formulation, and bioremediation (e.g. [60]), including its potential for use as extractive species in Integrated Multi-Trophic Aquaculture (IMTA) [61,62]. These species exhibit high plasticity and adaptability to environmental and growth conditions, which can strongly modify their quality [63]. They can proliferate rapidly [64] due to their high ability to uptake nutrients (nitrogen and phosphorus), and their high rate of photosynthesis [65]. Due to this ability, some *Ulva* species can sometimes cause kilometer-scale blooms termed green tides under eutrophication condition, where the seaweed

Table 4
Summary table for nutrient uptake efficiency (NUE) and nutrient uptake rate (NUR), all data are presented as average ($n = 3$) ± standard error and as percentage for NUE and as µmol g^{-1} WW h^{-1} for NUR for each treatment where: LW (lagoon water), LD (lagoon water enriched with digestate), LF (lagoon water enriched with F/2), LU (lagoon water enriched with sea urchins wastewater). Lowercase letters indicate significant probability level after the Monte Carlo test (PERMANOVA, $p < 0.05$).

Culture media	NUE DIN	NUE Nitrate	NUE Nitrite	NUE NH4	NUE Ptot
LW	−26.30 ± 25.62 ^{b}	24.742 ± 6.749 ^{b}	−27.020 ± 16.460	−44.411 ± 40.629	92.587 ± 0.441 ^{b}
LD	58.16 ± 8.95 ^{a}	28.261 ± 5.020 ^{b}	83.176 ± 8.631	55.144 ± 10.919	85.896 ± 3.932 ^{ab}
LF	32.96 ± 3.28 ^{ab}	94.160 ± 1.629 ^{a}	−374.167 ± 256.26	0.200 ± 23.948	96.214 ± 0.710 ^{a}
LU	19.14 ± 10.93 ^{ab}	92.832 ± 1.202 ^{a}	12.598 ± 6.860	−52.158 ± 33.464	96.107 ± 0.542 ^{a}
LW	0.166 ± 0.079	0.006 ± 0.001 ^{b}	−0.004 ± 0.003 ^{b}	−0.050 ± 0.073	0.020 ± 0.002 ^{b}
LD	0.624 ± 0.157	0.014 ± 0.003 ^{b}	0.020 ± 0.003 ^{a}	0.363 ± 0.133	0.027 ± 0.009 ^{b}
LF	0.809 ± 0.232	0.612 ± 0.192 ^{a}	−0.070 ± 0.047 ^{ab}	0.067 ± 0.192	0.157 ± 0.030 ^{a}
LU	0.381 ± 0.060	0.212 ± 0.010 ^{a}	0.003 ± 0.002 ^{b}	−0.104 ± 0.079	0.098 ± 0.007 ^{a}

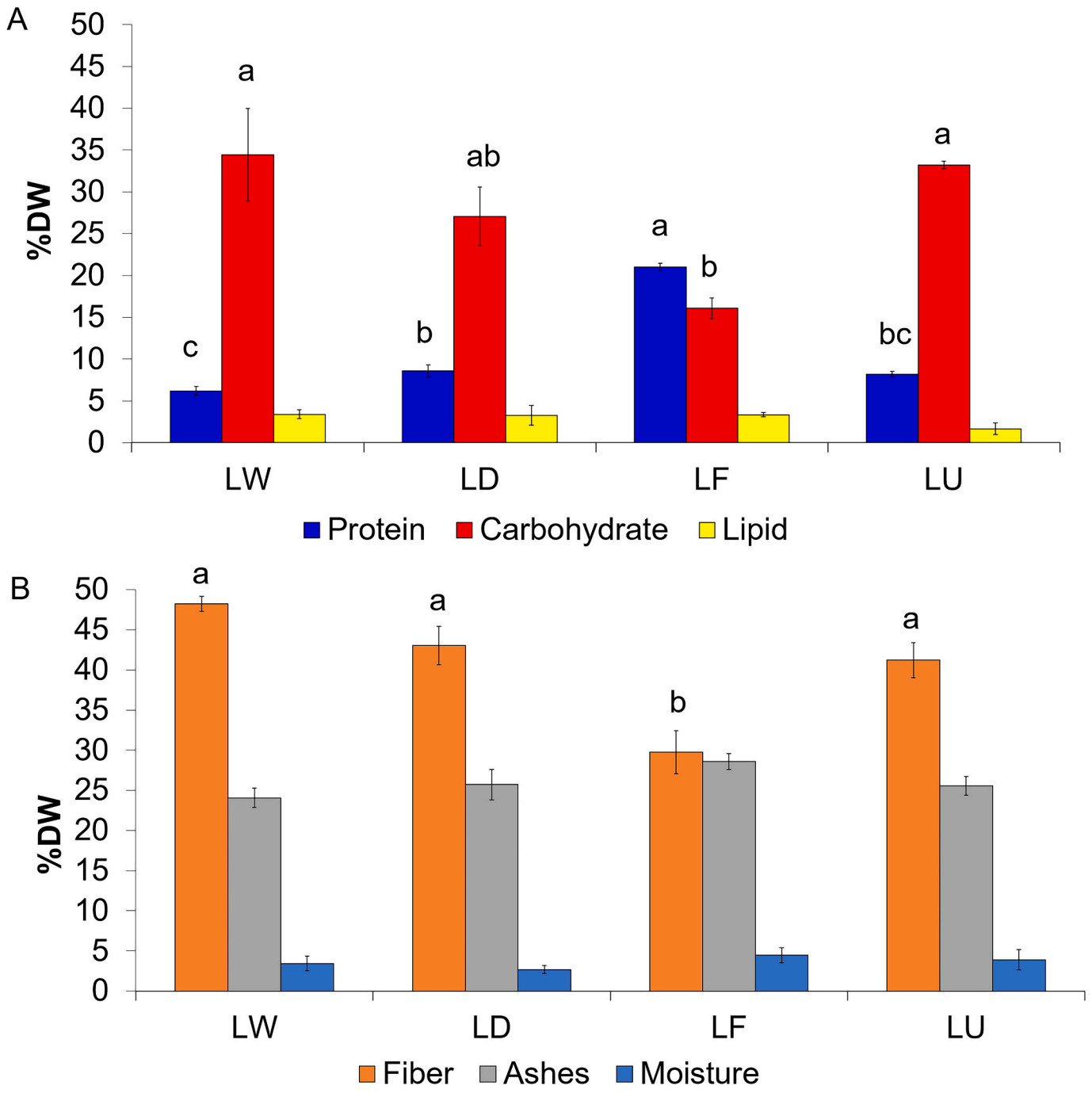

Fig. 4. A) protein, carbohydrates and lipid content (as %dry weight, DW); B) fiber, ash and residual moisture (as % DW). LW (lagoon water), LD (lagoon water enriched with digestate), LF (lagoon water enriched with F/2), LU (lagoon water enriched with sea urchins wastewater). Letters above bars indicate significant differences (Tukey's post-hoc test) only for significant variations. Error bars indicate standard error ($n = 3$).

increase its biomass in a free-floating state by increasing the size of the thalli and their fragments [66,67].

In this study, we have compared the growth, physiology, morphology, nutrient uptake, and composition of *Ulva* sp. grown under four different media. We have used as baseline lagoon water (LW), to understand the potential for growing the biomass without the addition of nutrients using the resources available, and we have compared it with two enriched wastewaters, one representing a waste from aquaculture activities (LU) and one farming waste (LD) and with one artificial culture media (LF) added to the LW. This in order to understand the nutrient removal capacity of the species subjected to different N and P sources and concentrations for application to a circular and sustainable blue economy model and the variation in composition, according to the media, which will subsequently determine applications of the biomass produced.

4.1. Growth assessment

The SGR recorded was higher for the biomass grown under F/2 (9.86 % day^{-1}), with this value being similar to the highest growth rate reported in the literature, particularly for those grown in tank systems, [19], for which growth rate between 10 and 50 % was considered exceptional (e.g. *U. prolifera* [7] and *Ulva fasciata* [68]). This suggests that the trialed cultivation system and conditions were ideal to

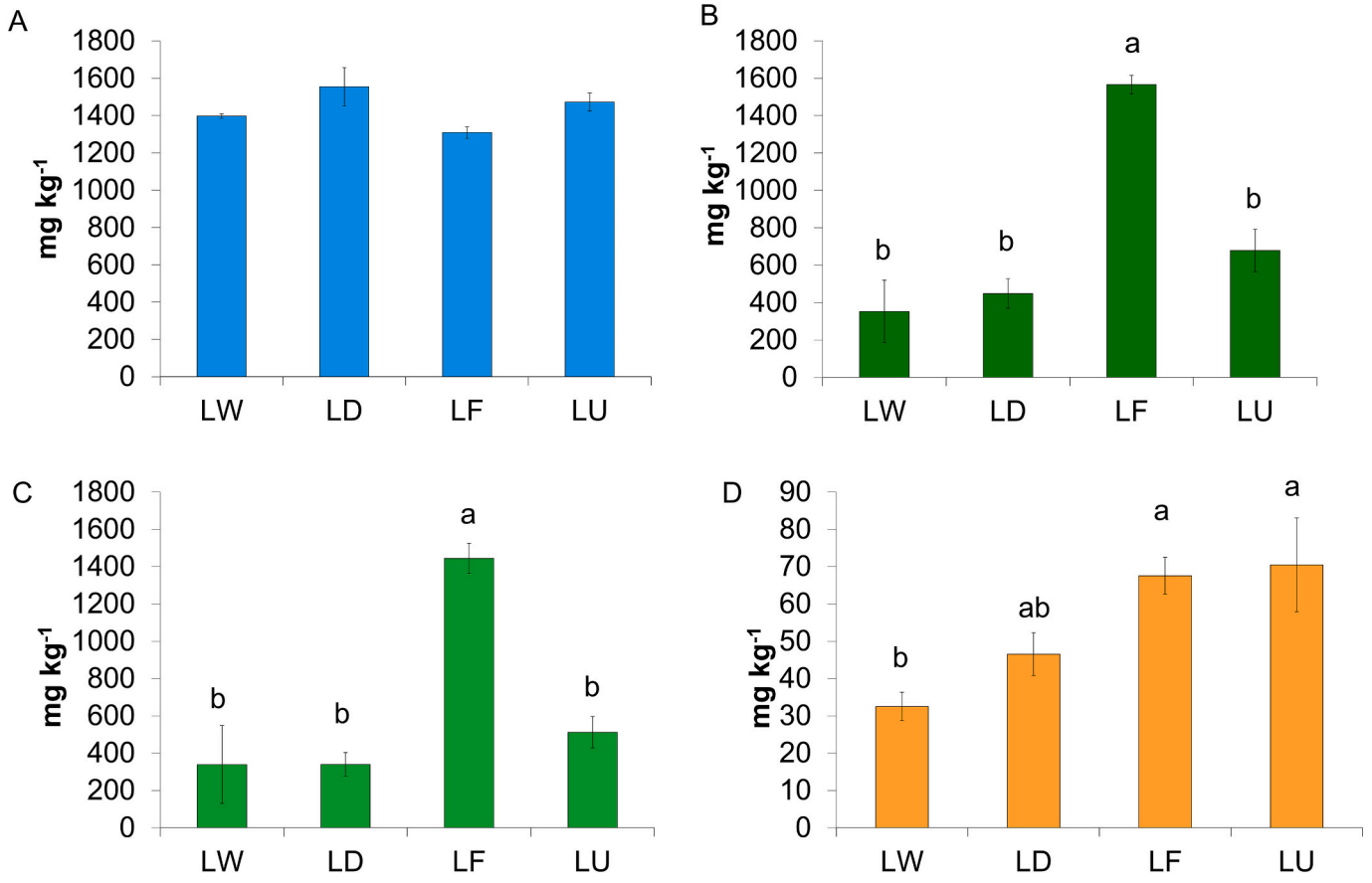

Fig. 5. A) Polyphenols, B) Chlorophyll-a C) Chlorophyll-b, D) Carotenoids of the biomass under different culture media (LW (lagoon water); LD (lagoon water enriched with digestate), LF (lagoon water enriched with F/2), LU (lagoon water enriched with sea urchins wastewater). Letters above bars indicate significant differences (Tukey's post-hoc test). Only significant variations are reported. Error bars indicate standard error (n = 3).

guarantee efficient biomass production in a short time interval (7 days) using the F/2 culture media. The highest growth of *Ulva* sp. was reported in F/2, followed by media with digestate, sea urchin wastewater, and finally lagoon water. This underlined the preference of the species for media with a high quantity of nutrients, presenting both nitrate and ammonia, which results in higher performance (e.g., [69,70]). These findings also show that under high nitrate concentrations, phosphorus uptake is not limited in *Ulva* sp. as previously reported for other sea lettuce species [71].

Changes in stocking densities and seasonal variations can have a significant influence on seaweed production in tank and mariculture systems with an impact on the relevant industries [72]. However, this simple and scalable photobioreactor system provides precise control over temperature, nutrient management, and efficient light transmission, and could be applied for year-round supply of consistent biomass, exempt from seasonal and temporal variations. This can incentive the development of an enhanced phyconomy and can enable seaweed cultivation in areas where weather and/or seashore are not conducive to cultivation, as already seen in previous studies that applied PBR systems [28].

4.2. Nutrient uptake and removal

Investigating the ability of seaweed species to absorb and utilize nutrients is fundamental to understanding their potential for downstream applications as bio-remediators and biofilters. Species of *Ulva* differ in their nutrient preference and their capacity to effectively use and assimilate them [19]. From our study, it appears that *Ulva* sp. is a valuable candidate for bioremediation/biofiltration, exhibiting similar biofiltration performance compared to other *Ulva* species [71]. The species was able to remove a considerable amount of nitrogen, particularly in the form of nitrate, ammonia, and phosphorous. Interestingly, when a similar or higher amount of nitrate was available compared to ammonia, the species showed higher uptake efficiency for nitrate, and vice versa when lower levels of nitrate were present compared to ammonia. This might suggest that the sources of nutrient preferences depend not only on concentration of the nitrate and ammonia themselves and the energy required to assimilate them (which is usually lower for ammonia, hence resulting often in the preferred sources, [7,68,73]), as previously reported for other species of *Ulva* (e.g. *U. lactuca* [65]), but it also depends on the initial ratio of the different sources present simultaneously.

The potential of digestate as an enrichment source for the cultivation of *Ulva* sp. was investigated with the perspective of utilizing the produced biomass for feed, biomaterials, biofiltration, and/or energy. It has been reported that some forms of N target increased growth, while others increase the tissue N content in seaweeds [74]; however, in this case biomass grown under higher concentration of nitrate (compared to the other sources of N) showed a simultaneous higher growth rate and protein content. Biomass grown in digestate showed similar growth rate and absorbance to sea urchin wastewater, and this enriched media had the highest initial nitrate and ammonia content of all the media tested. However, the highest total bioremediation efficiency was observed among the algae grown in F/2, as reported in previous studies for *U. lactuca* [75]. The cultivation of *Ulva* sp. with digested cow manure as a nutrient source has the potential to transform N and P into seaweed biomass that could find application in protein-feed, biomaterials and/or energy, with additional studies that could look into the metals and

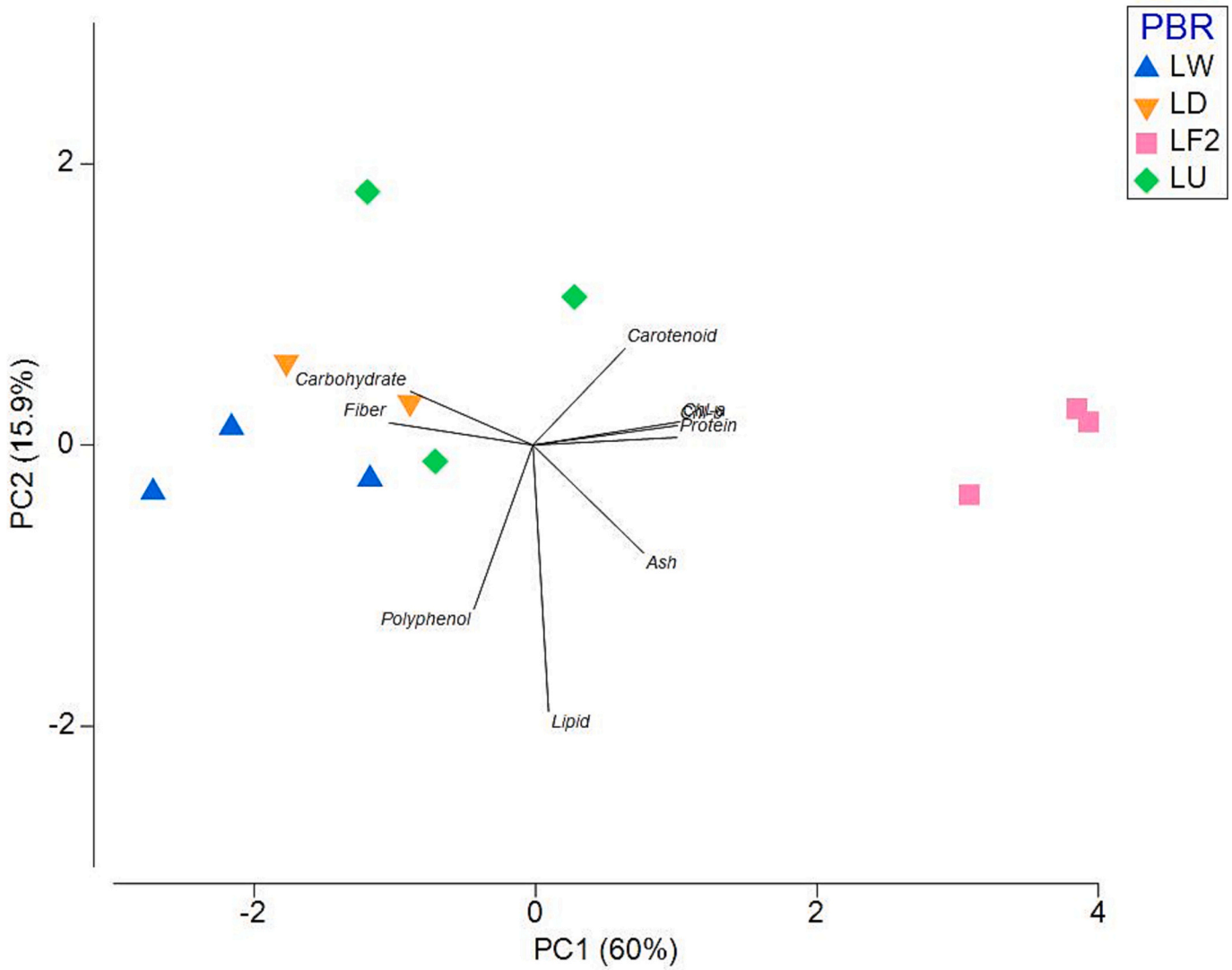

Fig. 6. PCA loading plot of PC1 and PC2 accounting for 75.9 % cumulative variation. The different colors identified the four culture media: LW (lagoon water); LD (lagoon water enriched with digestate), LF (lagoon water enriched with F/2), LU (lagoon water enriched with sea urchins wastewater). PBR refers to the 4 different treatments in photobioreactors.

minerals composition of the grown biomass and the overall health safety of the biomass produced. Phosphorus limitation was not recorded during the trial and the species was able to efficiently uptake it and use at the same time of either nitrate (LU and LF) or nitrite (LD), depending on their initial concentration, with overall level of removal for P even higher than the nitrogen sources (Table 1, Supp. Table 1 and Fig. 3), even in LW where N sources were scarce. This showed that N and P uptake are independent, as already seen for other species of *Ulva* [76] but the presence of nitrate facilitated P uptake, as already seen for other *Ulva* species that likely use P for the production of essential cofactors for the reduction of nitrate to ammonia prior to assimilation [68]. Different assimilation rates of nutrients are typical in algae and it has been previously reported that among the three groups of seaweeds, green seaweeds tend to grow more, be more efficient, and accumulate more nitrogen while grown in wastewater compared to the brown and red seaweeds [77]. High performance of the species in wastewater treatments was identified also in this study, suggesting that digestate and sea urchin wastewater could be valuable alternatives to LF, depending on the final biomass application.

Another important parameter that affects growth and productivity in seaweeds is the N:P ratio, with N limitation being the predominant factor to growth limitation in green seaweeds. While this ratio varies between species, the optimal ratio for seaweeds has been identified as 30:1 [78]. In this study, the N:P at T0 was below the optimal levels for all media (2.6–11); however, at T*final* this was included between 34.6 and 119.6 reflecting the efficiency of the system and the physiological response of the biomass.

Although various wastewater sources have been widely investigated either independently or in IMTA systems for seaweed cultivation including species of *Gracilaria*, *Ulva*, and *Laminaria* (e.g. from fin fish, shrimp, microcrustacean, polyculture with clams, abalone, and sea squirt, [77,79,80]), and polyculture of *U. lactuca* with fish and sea urchins has been previously reported [31], there is no information available in the literature on the direct application and effect of sea urchins wastewater applied for seaweed cultivation, and this is the first time this has been trialed in a PBR system. Biomass grown under this medium in this trial was third for growth performance and the second best performing in NUR and NUE. The initial content of phosphorus and nitrate in the media was second only to F/2 (Table 1) and the biomass showed high polyphenols and carbohydrates content (Fig. 4). These preliminary results suggest that sea urchin wastewater is idoneous for *Ulva* sp. cultivation in the photobioreactor system trialed, resulting also in enhanced quality of biomass for valuable compounds such as carbohydrates and polyphenols. Application of this medium would be

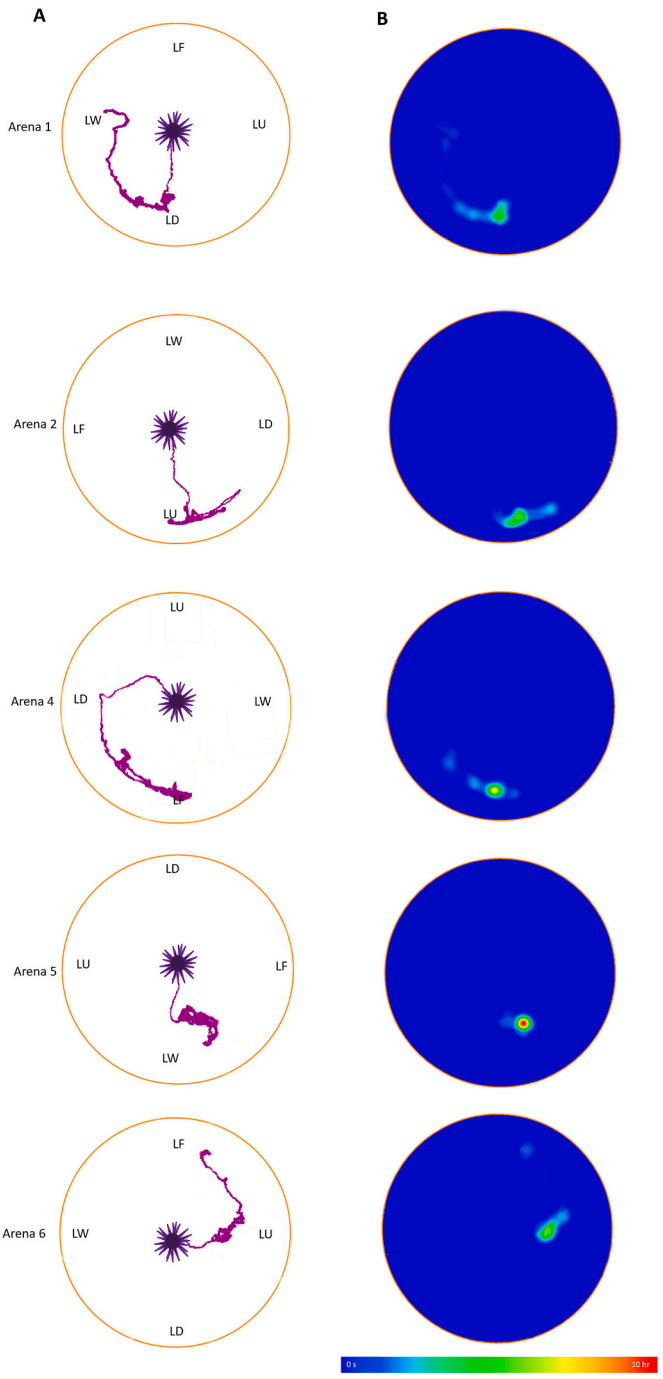

Fig. 7. Trajectory A) and heat map B) of the sea urchins in the trial arenas obtained with ANY-maze tracking software. Arena 3 was only analyzed manually because the video was not suitable for the software analyses. LW (lagoon water), LD (lagoon water enriched with digestate), LF (lagoon water enriched with F/2), LU (lagoon water enriched with sea urchins wastewater).

particularly valuable in the blue and circular economy. If the biomass was to be grown for feed purposes, considering the growth performance, biochemical composition, and the results of the palatability trial obtained in this study, *Ulva* sp. cultivated using this nutrient and then used as feed for the same species (*P. lividus*) or for other invertebrates, will constitute a cost-effective and circular system. This would require limited input of nutrient for seaweed production and therefore reduction of costs associated with it, improving the sustainability and waste management of the system. Hence, further investigation should be undertaken to exploit the potential of this production chain system that fits in the perspective of the circular economy and seaweed contribution to SDGs [59] such as responsible consumption and production and life below water.

4.3. Morphology and physiology

Seaweeds are fundamental primary producing organisms that have different absorption properties which will influence their ability to capture light and consequently affect their growth and productivity [81]. The fraction of incident light absorbed by seaweeds depends on various characteristics such as size, shape, and thickness of thallus and it appears to be closely correlated with their pigment concentrations [82,83]. For species whose thalli already absorb the majority of incident light, the absorption is unrelated to the pigment level [82,84,85], which reflects what was observed in this study. The absorbance was correlated with the pigment content and varied according to the media and the color of the blade. Darker blades, such as those of the biomass grown under F/2 and sea urchin wastewater, corresponded to higher absorbance levels (0.40 and 0.38, respectively). Lower levels were instead associated with digestate and particularly water lagoon alone, for which the biomass appeared paler (Supplementary Fig. 6).

Previous studies have reported variations in seaweed thallus color corresponding to variations in nitrogen content for other species of *Ulva* including *U. lactuca* and *U. fenestrata*, where to a darker green color corresponded higher nitrogen and chlorophyll content [77,86–89] as reported in this study, with recent studies looking also at additional compounds that might be linked to variations in color (e.g. lipid and amino acid) [24]. Nevertheless, the color assignment is often subject to the sensitivity of the observer which can lead to different color perceptions. The potential of using the color as a composition proxy has been trialed and proved via photo RGB image analyses for *U. fenestrata* [90] and recently using CIELab coordinates [24]. The analytical method using the CIELAB color space, applied here on *Ulva* biomass, prevents a biased way of defining the color by applying instrument-calculated coordinates expressed as lightness, redness, and yellowness [24,91]. In this study, strong correlations were reported between a* and L* and the protein content, where darker and greener biomass corresponded to increased protein content. The application of the color meter in this study underlines the potential for a tool still underexplored that could help create color guides for the estimation of protein content, and other valuable compounds, based on accurate color variations, developing a database for fast and intuitive biochemical content assessment in *Ulva* sp.. As already seen in other studies, color is a powerful parameter to estimate seaweed nutritional profile, and the potential for this application has already been investigated and proved.

4.4. Biochemical composition

The biochemical composition of seaweeds is important for any application that aims to provide high-quality and consistent biomass for food and feed products. The biomass composition is often affected by several parameters, such as light, temperature, salinity, and nutrients [92]. The media investigated significantly affected the overall biochemical composition of the cultivated biomass. The protein content reported was in the higher range compared to other studies for similar species of the same genera [77,93]. It has been shown that increased nutrients and temperatures result in higher total protein content of *Ulva* spp. (e.g. [94–97]) where high initial levels of nitrate in the media contribute to increased protein synthesis [96]. Whereas protein and nitrogen content in seaweeds will be reduced when nitrogen is limiting [98]. The combination of temperature and nutrients tested in this study (particularly using F/2) suggests ideal conditions to target protein production in *Ulva* sp. in this photobioreactor system.

It has been previously reported in seaweed that high levels of protein correspond to lower levels of carbohydrate [98,99], and this was confirmed also in this study. The carbohydrate content reported in this

study (16–34 %) was in the lower range compared to previous studies on other species of *Ulva* that reported content between 40 and 64 % [100–102]; while similar content of NDF (30–48 %) was reported compared to other *Ulva* species [101,102] that suggests a favorable nutritional profile for humans and animals.

Compared to other species of *Ulva* investigated in cultivation trials (e.g. 0.2–1.6 %, *U. rigida* [23]), this study reported higher content of lipid (1.7–3.4 %), similar to what previously reported for *Ulva* sp. grown in IMTA [103] and land base systems [104], underlying the influence of the cultivation conditions (particularly using enriched media) on the fat content of *Ulva* sp.. It has been previously reported that the lipid content is also influenced by temperature, with lower temperature usually resulting in higher lipid content [23,105]. However, results from this study point towards a different trend considering that the temperature selected for the experiment was 20 °C, hence on the higher range, suggesting that other metabolic processes of storage mechanisms and/or triggered synthesis might be at play. While lipids are usually low in seaweeds, although higher in green algae compared to brown and red [106], they represent valuable compounds with important downstream applications, and the content assessed in this study for *Ulva* sp. is promising. Further analyses should look at the fatty acid profiles of the species to understand the potential for commercial applications.

Other commercially interesting compounds present in seaweeds are polyphenols. There is an increasing demand for natural antioxidant molecules that could represent alternatives to synthetic additives in the food industry. Phenolics are valuable compounds with antibiotic and antioxidant activity [107] that are sought after in cultivated seaweed biomass due to their potential for applications in foods and cosmetics [108]. It has been previously reported that high nutrients coupled with natural sunlight are ideal to increase phenolics and antioxidant activity in other species of *Ulva* (e.g. *U. fasciata* [109]). The total polyphenol content reported for *Ulva* sp. was similar or higher compared to previous studies on related species (e.g. *U. rigida* [109]) while pigments values, including Chl-a, Chl-b and carotenoids, were within the range already reported for other species of *Ulva* [109,110]. Pigments have recognized applications as food and textile dye, in the cosmetics and pharmaceutical industry [111]. Preliminary results reported in this study suggest that further analyses are required to understand the potential to exploit this species for extraction of antioxidant compounds with valuable downstream applications and suggest potential benefits for consumers (human and/or animal) due to the presence of these compounds.

4.5. Palatability preference trial with sea urchin (*Paracentrotus lividus*)

Like other marine invertebrates, sea urchins exhibit feeding preferences based on food availability, optimal use of diet components and the characteristics of each seaweed species available, particularly morphology, nutritional properties and chemical defenses [112]. Feeding preference trials, like the preliminary study here proposed, can generate interesting comparative information regarding diet preference and consumption and can provide insight into optimization strategies in aquaculture applications. Sea urchins rely on chemical senses to detect and localize food resources and they have been reported to respond to distant feeding stimuli using chemtrail navigation and odor source localization, which can be used in feeding preferences and chemosensitivity studies [113]. The use of cultivated seaweed for animal feeding is raising interest with particular interest for their nutritional value and as a feeding stimulant, able to foster the food intake and increase the conversion rate of the reared animals. The knowledge of the abilities of the sea urchins to detect and respond to feeding cues that can have attractant and/or phagostimulant activity, like the ones trialed here, can have strategic applications in developing effective aquaculture feeds. For a diet to be effective, it is fundamental not only that the formulation is nutritional but more importantly that the animal can locate it, choose it and consume it [113]. An example is the use of sea lettuce for the grow-out of reared sea urchins, as a feeding itself or as ingredients in prepared diets [15,114,115]. *P. lividus* is nowadays considered overexploited in many regions, and reared juveniles can be used for restocking activities [116] or commercial production. The use of *Ulva* sp. in the diet can increase the quality of the gonads [15]. This sea urchin species is a model species used in feeding and chemostimulant bioassay [113,117,118]. Sea lettuce is significantly attractant and evokes a clear and strong physiological response in this herbivore [12]. Moreover, it has been shown that sea urchins have a selective and dose-dependent response to some sugars and amino acids, giving information on the sensitivity of the species to food-related compounds [12,118]. In the present experiment, the sea urchins preferred the biomass produced with recycled water nutrients, in particular sea urchins wastewater (LU) followed by digestate (LD). As counterintuitive as it might be, sea urchins were not strongly attracted by the biomass with higher protein content, but instead, by the biomass with higher carbohydrates, fiber, and polyphenols content. Polyphenols have been found to have mixed effects on other species of invertebrates, including sea urchins, acting also as stimulus for enhancing feeding [119]. We hypothesize that this result might be due to the possible higher content of ulvans, sulphated polysaccharides, usually soluble in water and composed of rare molecules like rhamnose and uronic acids, which are helpful in food supplements and biomedical applications and present in both cell wall of filamentous and foliose *Ulva* sp. biomass [120]. While this was a preliminary investigation rather than a conclusive study, it provides valuable insights that can guide future research and provide a new tool to study the feeding behavior of sea urchins that can complement the traditional food consumption rates and stomach contents analyses [15,16,121,122]. Sea urchins are known to exhibit selective feeding behaviors based on the nutritional content and chemical defenses of algae. The observed trends in this trial, even if based on a small size sample, highlighted for the first time new cues and ways in which *Ulva* can be grown for a suitable and sustainable diet for the species. Additionally, the experiment contributes to our understanding of how different growth media affect the palatability and preference of *Ulva* to *P. lividus* with further aquaculture implications.

5. Conclusion

The performance of *Ulva* sp. produced using four different culture media was assessed and the nutrient uptake potential of the species under the four different nutrient composition was evaluated. It was found that the media influenced all the parameters examined. *Ulva* sp. grown in higher nutrient content overall showed faster growth, higher absorbance, protein, polyphenols, and pigments content, underlying the valuable potential for this biomass to be used for downstream applications. These characteristics points towards applications of the cultivated biomass for human food, cosmetics and pharmaceutical, markets for which a certain profile and quality of the biomass produced is required and that are profitable, justifying the increased cost of the biomass produced in the PBRs and particularly with the enrichment of the F/2 nutrients. Lagoon water alone resulted in the lowest growth rate and absorbance, although showing the potential for culturing the species without the addition of nutrients directly in the lagoon and/or seashore environments, for example as bioremediation/biofilter in polyculture. This could potentially lead to the production of biomass that can find valuable applications as biomaterial or for extraction of polysaccharides such as ulvans, that can be used in biomedical applications and as a source of antioxidant, and pharmaceutical compounds. Further analyses should look at minerals and metals composition in similar systems to understand potential for contaminants. The PBR system tested here proved to be efficient in ensuring significant growth of biomass in a short time interval, ensuring controlled cultivation conditions, exchange of gas and nutrients, and idoneous amounts of light. This system could represent an alternative cultivation methodology for the species, particularly if the final biomass can find applications as high-value products that justify the additional investment. Moreover, this study

underlined the potential for a blue and circular economy for the development of systems that can use discharge from other processing (e.g. digestate) and aquaculture activities (e.g. sea urchins farming) as a resource for biomass production (e.g. seaweed cultivation) and in addition bring this product back into the production chain and use it as valuable invertebrate feed, for a sustainable and cost-effective model.

Author statement

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

CRediT authorship contribution statement

Viviana Pasquini: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Cecilia Biancacci:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Massimo Milia:** Writing – review & editing, Formal analysis. **Davide Moccia:** Writing – review & editing, Formal analysis. **Paolo Solari:** Writing – review & editing, Visualization, Formal analysis. **Alberto Angioni:** Writing – review & editing, Formal analysis. **Pierantonio Addis:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors report no commercial or proprietary interest in any product or concept discussed in this article.

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All experimental procedures are fully compliant with the European Directive 2010/63/EU concerning the animal protection during the scientific research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103749.

Data availability

Data will be made available on request.

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