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Cultivation of Autochthonous Microalgae for Biomass Feedstock: Growth Curves and Biomass Characterization for Their Use in Biorefinery Products

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Abstract: In this work, the biomass productivity for biorefinery products and growth curves of three autochthonous microalgae collected in different reservoirs ("*Scenedesmus* sp." (SSP), mixture of *Scenedesmus* sp., *Chlorella minutissima*, *Chlorellas* sp. and *Nannochloropsis* sp. named "*La Orden*" (LO) consortium and *Chlorella minutissima* named "*Charca Brovales*" (CB) consortium) were studied in a 5.5 L column laboratory photobioreactor. Two different culture media, Arnon culture (AM) and an agriculture fertilizer-based liquid medium (FM), have been used to evaluate the growth effect of the microalgae; it was found that the medium has a clear effect on the biomass productivity and growth rate, which ranged between 0.26–0.498 g L^{-1} d^{-1} and 0.288–0.864 d^{-1}, respectively. In general, the elemental analysis and higher heating value of microalgae biomass for the three species were independent of the culture medium used for its growth, while their lipids and sugars content depended upon the species type and culture medium used in the cultivation. "*La Orden*" microalga was selected (given its best adaption to the climatic conditions) to study the biomass productivity and growth rate in two exterior photobioreactors (100 L column and 400 L flat panel), using FM as a medium, obtaining values of 0.116–0.266 g L^{-1} d^{-1} and 0.360–0.312 d^{-1}, respectively. An automation and control system was designed to operate the exterior photobioreactors pilot plant. The lipid content of this microalga in these photobioreactors was lower than in the laboratory one, with a fatty acids profile with predominantly palmitic, oleic, linoleic and linolenic acids. Also, the fresh biomass collected from these photobioreactors was studied in a batch type digestion process for biogas production, obtaining a CH_4 yield of 296 ± 23 L CH_4 kg_{VSS}^{-1} added with a reduction in percentage of COD and vs. of 50 ± 1% and 50 ± 1.7%, respectively.

Keywords: autochthonous microalgae; culture medium; biorefinery; biomass production; biofuels

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1. Introduction

The 2030 climate and energy framework sets targets for cutting greenhouse gas emissions and increasing the share of renewable energy and energy efficiency [1]. Under the energy union, the EU is working to integrate Europe's energy markets, ensure energy security, improve energy efficiency and decarbonize the economy. In this sense, in the last years, the biorefinery concept has been implemented. According to this concept, a system must guarantee a unified approach in the generation of valuable materials and biofuels via the integration of biomass conversion processes [2]. A quite complete review has been published describing the possible conversion technologies to transform meat processing waste into biochemicals and biofuels [3]. In this line, alternative renewable

processes to produce energy carriers as algae or other plants have become a hot topic for research [4]. Currently, the bioeconomy of the processes and products based on microalgae faces serious restrictions [5]. In this context, Depra et al. [5] have published a recent and very interesting review on "Microalgal Biorefineries for Bioenergy Production: Can We Move from Concept to Industrial Reality?" establishing the advantages and benefits of the production of biofuels, bioenergy, biorefinery chemicals and bioproducts from microalgae as feedstock.

Liquid biofuels are considered sustainable and carbon-neutral alternatives to petroleum-based fuels. Among all the potential feedstock for biofuels, microalgae stand out due to their significant advantages such as high growth rate, less competition with food production and lower environmental impact when are compared with other biomass precursors. As energy feedstock, microalgae biomass can be transformed into many types of biofuels: biodiesel, biohydrogen, biogas or it may also be used for direct combustion [6–11]. Recently, many research studies defend that algal biofuels would be a potential alternative to petroleum-derived fuels in the transportation sector [6,8,12–14].

Microalgae have received notable attention because of their high photosynthetic rate (approximately 50 times higher than that of terrestrial plants). Recent studies have reported photosynthetic rates of 6.9×10^4 cells mL^{-1} h^{-1}, based on the *Chlorella vulgaris* cell number of 5.7×10^7 cells mL^{-1} for 34 days in control media [15].

In addition, microalgae contain high amounts of lipids, proteins and carbohydrates, which can be used for different applications [16,17]. However, a large amount of energy is needed for algae biomass collection and dewatering, which in turn generates various pollutants/emissions [16–21].

Despite this issue, microalgae remain imperative as a future energy feedstock, as it requires less land compared to other commercial crops such as palm or jatropha [22].

Since one major factor influencing microalgae productivity is genetics, the selection of microalgae for cultivation is important [23]. Typical culture methods for increasing the productivity of microalgae are: (a) two-stage culture (manipulating the culture conditions and nutrient feed in terms of the period and concentration to increase the cell reproduction rate and lipid content of the microalgae); (b) phototropic culture (microalgal cells depend on light to reproduce) and (c) heterotrophic culture (it consumes other organisms or organic waste containing carbons as an energy source instead of CO_2 and is independent of the light source for reproduction). The most important factors influencing microalgae productivity are the concentration of dissolved oxygen and carbon dioxide in the medium, light conditions, nutrient supply and source, pH and climate (temperature) [23,24].

Both open or closed systems can be used for microalgae cultivation, and the choice of the type of system will have a significant effect on the production cost of biofuel. For instance, cultivation in an open system (pond or tank) involves a greater degree of exposure to the environment, while a closed system has less contamination and can be more easily controlled. Open ponds can be classified as raceway, circular, inclined and unmixed ponds. On the other hand, different reactor configurations can be used for cultivation in a closed photobioreactor, such as tubular, vertical, flat-plate, annular, fermented-type and internally illuminated photobioreactors [23,24].

Estimations report that biodiesel production depends on the content of TAG (triglyceride), which corresponds to more than 70% of the lipid content [25], as well as the biomass productivity. The biodiesel production depends on the TAGs concentration in the lipid content of an oil or fat. According to the transesterification reaction, the ratio of the feedstock mass and biodiesel produced is 1:1, which occurs in a reactor where the blended alcohol and catalyst react with TAGs of the oil at a temperature close to the alcohol boiling point [26–28]. Yun et al. [10] have published the effect of flue gas CO_2 on growth, lipid production and fatty acid composition of a green microalga *Acutodesmus obliquus*. El-Sheekh et al. [11] have observed that the distribution of fatty acids in the oil from *Chlorella vulgaris* varied under different conditions of culture stress.

The ethanol production from microalgae is primarily obtained through the fermentation of the starch, sugar and cellulose contained in microalgae biomass [29]. In this way, the conversion of biomass from marine algae into ethanol could be economically feasible since some algae hydrolyzates can contain more total carbohydrate and hexose sugars than some terrestrial lignocellulosic biomass feedstock [29–31]. It has been reported that the carbohydrate content of a marine macroalga (*Enteromorpha species*) is in the range 70 to 72% [32], where starch dominates the carbohydrate content and can be up to 60% (dry basis) for *Chlorella vulgaris* depending on the culture conditions [33]. This starch content of microalgae can be improved by controlling the N or Fe during cultivation [34,35].

According to the new concept of biorefinery [2,3,5], another possibility of exploitation of microalgae biomass is biogas production from anaerobic digestion. Some works have been reported in the literature with excellent results [7,36], and biogas production for different microalgae biomasses has been included in a recent review [8]. Literature data indicate that conversion of the algae biomass into biogas is a highly profitable solution and the obtained methane achieves 140–360 mL/g volatile solids (VS) depending on the algal species (*Chlorella sp.* and *Micractinium* sp.) [9].

This work presents a study of the microalgae growth and biomass production of three species: one control species which was *Scenedesmus* sp., and two autochthonous species, one collected from a water reservoir (named "*Charca Brovales*") where the species *Chlorella minutissima* is predominant, and other specie constituted by a mixture of *Scenedesmus sp*., different *Chlorella* species and *Nannochloropsis sp.* (named "*La Orden*") collected from different water reservoirs in the Extremadura region (SW of Spain). The research has been carried out with two different culture media, Arnon medium [37] and agricultural fertilizer based medium, in three different photobioreactors (5.5 L laboratory column, outer 100 L column and outer 400 L panel). The growth curves, kinetics and biomass production have been analyzed. Also in this work, according to the biorefinery of microalgae biomass, the possibilities of bioenergy and biofuels (biodiesel and bioethanol) and biogas production were analyzed and related to biomass precursors (in particular, to their lipids and sugars content).

2. Materials and Methods

2.1. Species Studied and Culture Conditions

The *Scenedesmus sp.* control species was supplied by the research group of Prof. Acién, from Almeria University (SE of Spain).

The autochthonous species named "*Charca Brovales*" and "*La Orden*" were collected from different water reservoirs located in Extremadura region (SW Spain), and are a "consortium" of different strains. The "*Charca Brovales*" consortium was collected from a water reservoir (named Charca Brovales, ambient temperature ranges from 3 °C in winter to 33 °C in summer, mean salinity is 4.5 ± 0.2 g/L) located near to a metallurgy industry at Brovales village (SW of Extremadura, Spain). We have chosen these reservoirs because of the proximity of important industries that can use them for their cooling systems and possible discharges, such as a nuclear power plant (Arrocampo), or a steel industry (Charca Brovales). The consortium named "*La Orden*" is a hybrid of *Scenedesmus* sp. and other microalgae species collected in two water reservoirs: Charca Brovales and Arrocampo (this reservoir is near Almaraz Nuclear Plant, and its water is used for the refrigeration system of this plant, ambient temperature ranges from 6 °C in winter to 29 °C in summer, mean salinity is 6.7 ± 0.2 g/L).

Isolation, selection, conservation and description of autochthonous microalgae were as follows: the samples were harvested and taken to the laboratory, where they were filtered (6 μm paper) to remove suspension partícles (such as fallen leaves, gravel, etc). Subsequently, the growth of the species was carried out with the *Arnon* medium [37] in glass 2 L photobioreactors, previously sterilized in an autoclave. The culture conditions were: ambient temperature in the range 20 ± 1 °C, artificial light with total intensity of 1600 μmol photons m^{-2} s^{-1} and photoperiod of 12 h light/12 h dark. The pH ranged

between 7.5–8 and was maintained in these values by means of the addition of pure CO_2. The samples were kept in culture mode until the microalgae proliferation was observed; in those cases, in which no proliferation was observed after 15 days, the samples were discarded.

Once the growth of the cultures was detected, these were subjected to regular injections of highly pure CO_2 flows; it was always controlled in the range 7.5–8 as abovementioned, to guarantee higher biomass productivity.

Afterward, the more abundant microalgae in the culture were observed by light microscopy, and then, they were isolated to obtain monoalgae cultures, pure strains and thus to carry out the research on the same material. Special care was taken to guarantee good conservation of the inocula, in case it would be needed for further culture stages. The method used to carry out the isolation was the culture technique at solid medium by extension in a Petri plate [38].

Two different culture media were studied: an Arnon medium (AM) and an agricultural liquid fertilizer based medium (FM) made from agricultural fertilizers, with the aim of reducing input costs. "Arnon medium" has been widely used in the cultivation of algae (mainly for freshwater species) and has shown good results in many microalgae species. Table 1 shows the composition of this medium. The most important nutrients are always nitrogen and phosphorus. These can also be supplied in the form of agricultural fertilizers, which are usually available, but it also implies an additional cost [39]. Another way of preparing the culture media consists of adding liquid fertilizers N P K and microelements well diluted in water that was previously autoclaved, in order to avoid contamination by rotifers and other predators. The agricultural liquid fertilizer medium (FM) used in this work was constituted by: 9 mM (millimolar) of nitrogen as nitrate (NO_3^-), 0.5 mM of phosphorus as phosphate (HPO_4^{2-}), 3.8 mM of potassium as (KOH) and 0.1 mM of microelements (solution of B (0.05%), Fe (1.03%), Mn (0.70%), Cu (0.12%), Mo (0.01%) and Zn (0.33%) in EDTA)) per liter of well water.

Table 1. Arnon medium composition.

Dissolution 1	Chemical Compound	Quantity(g L^{-1})	(mL L^{-1}) *
solution stock 1	Na_2MoO_4 2 H_2O	1.26	1
solution stock D7-(Mo-V)	H_3BO_3 $MnCl_2$ 4 H_2O $ZnSO_4$ 7 H_2O $CuSO_4$ 5 H_2O $CoCl_2$ 6 H_2O	2.86 1.81 0.222 0.79 0.403	1
solution stock 2	$MgSO_4$ 7 H_2O	124	1
solution stock 3	$CaCl_2$ 2 H_2O	15	1
solution stock 4	NaCl	117	1
solution stock Fe-EDTA	$C_{10}H_{16}N_2O_8$ KOH $FeSO_4 7H_2O$	16 g in 186 mL water 10.4 g in 186 mL water 13.7 g in 384 mL water	1
solution stock 5	$NaNO_3$ 2M	170	10
Dissolution 2			
solution stock 6	K_2HPO_4 1M	174	4

* volume of each stock solution added to 1 L of distilled water solutions comprising 1 and one liter of solution 2. Solutions 1 and 2 were autoclaved separately and joined to form the *Arnon* medium.

2.2. Photobioreactors

Experiments were carried out at temperatures between 20–35 °C with a photoperiod day: night from 12:12 h, provided by fluorescent tubes (14 Day-light Phillips 18W/865 providing a light intensity of 2000 µmol photons m^{-2} s^{-1} uniformly to the three high

performance photobioreactors). The 5.5 L methacrylate columns (diameter of 0.125 m and height of 0.5 m) contain a front output to obtain harvested algae, a bottom outlet for the total emptying (which in turn serves for the introduction of air), an upper plug and pH and temperature sensors. Commercial pure CO_2 was introduced on demand according to pH control between 7.5 and 8, the flow rate was set at 0.25 L min^{-1} and the air flow to bubbling at 1.5 L min^{-1}. All batch cultures were performed in order to determine the complete growth curve in all its phases. This experiment was conducted in the laboratory in different media, Arnon medium (AM) and agricultural fertilized medium (FM).

Subsequently, experimentation was extrapolated to a pilot plant, where the consortium "*La Orden*" was studied at FM at the concentrations described above. For this experiment, we used two photobioreactor models, which can be seen in Figure 1. One was a vertical column of 100 L capacity, consisting of an acrylic cylinder of 20 cm diameter and 2 m height. In this system, the mixture was obtained by injecting air into the bottom of the column, and CO_2; pH and temperature were monitored during operation by a pH meter and thermometer located at the top of the column. The other photobioreactor used in the study consisted of a flat structure made of a transparent plastic bag with a high surface/volume ratio, with an approximate capacity of 400 L. From the column photobioreactor it was passed to the flat photobioreactors that constituted the pilot plant of 10 photobioreactors. The cultures agitation was made by a bubbling system, consisting of a metal tube with small holes through which air and CO_2 run longitudinally to the bag bottom. The photobioreactor may be vertical or inclined. In the latter case, the inclination can help to delay the adherence of the microalgae cells in the upper wall of the photobioreactor, facilitating the solar radiation on the medium.

Figure 1. Vertical column and flat-panel photobioreactors.

2.3. Automation and Monitoring System of the Pilot Plant

In this section, the deployment of an automation and monitoring system for controlling the growing process of microalgae in both types of photobioreactors (column and flat panel) is presented. The main components, hardware and sensing features are described. The most relevant contribution of the implemented system is that all the tasks in the photobioreactors are performed in a fully automated way.

Among the available technology for process automation, Programmable Logic Controllers (PLCs) are the most suitable and versatile tools to carry out such tasks [40].

We have chosen this industrial control unit due to its features of reliability, robustness and stable operation, critical issues in the presented application given the severe conditions of the equipment to be controlled. Additional benefits are its small size and low power consumption. In addition, PLCs have digital and analog modules to connect the required sensors with proper measurement accuracy.

In the proposed approach, the controller measures and governs the different parts of the process by means of diverse sensors and actuators. Moreover, for monitoring purposes, a human–machine interface (HMI) is responsible for providing information to the operator of the plant in a user-friendly way. The real-time visualization constitutes a paramount function to achieve an effective tracking and assessment of the process behavior. These components are connected through a PROFINET (Process Field Net)-based network.

The automation system comprises a Siemens S7-1200 PLC equipped with analogue and digital Input/Output (I/O) modules. The models of these devices are now listed:

CPU 1214C AC/DC/Rly

HMI TP600 Comfort touch panel

SIMATIC ET 200S scalable system for distributed automation

This PLC controls the feeding of required CO_2 and the corresponding filling and harvesting cycles of the bioreactors. In addition, as aforementioned, this system fulfils the role of data acquisition. Data are displayed and stored on the KTP600 touch panel, where the SCADA (Supervisory Control and Data Acquisition) application is executed. The touch panel logs the variables of interest at one minute intervals from the PLC's memory by a permanent connection. All the variables can be plotted and displayed in real time, with the touch panel allowing remote access to the data via Internet. Since these plots provide real-time information about the functioning of each of the subsystems, they allow the researcher to monitor the operation of the system under the program.

In order to obtain the measurements of the process variables, a set of sensors were installed in the bioreactors of the pilot plant. The most important variable to be measured is pH, as the CO_2 injection depends on its value. This measurement was carried out by a pH/EC transmitter HANNA HI 98143. As said before, temperature also has an important role in the growing process of microalgae, so this variable is measured by a thermoresistor, PT100. Both sensors, pHmeter and PT100, are connected to the PLC through a decentralized periphery station ET 200S, which is connected to the PLC using a field bus PROFINET protocol. With this station, data from temperature and pH sensors are acquired and sent to PLC memory positions. Figure 2 shows the block diagram of the automation system.

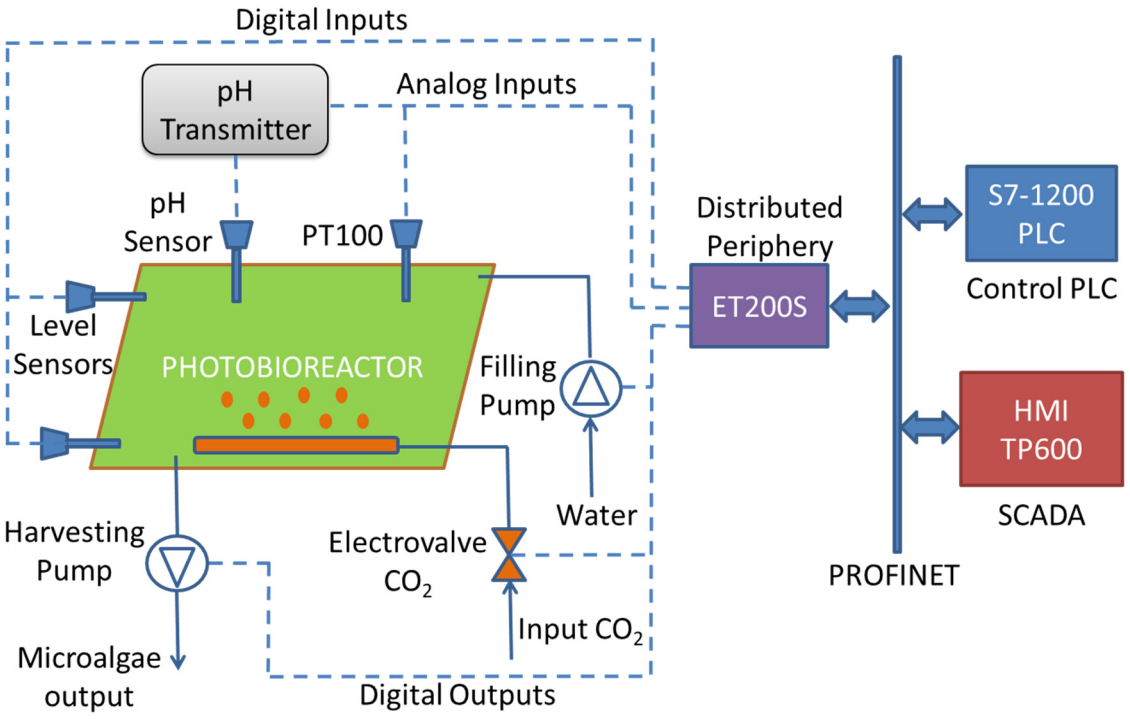


Figure 2. Automation and monitoring system of the pilot plant.

From the measurements obtained of sensors and signal conditioners, the control system will determine the switching conditions of the valves that introduce CO_2 in the bioreactors. These valves are connected through digital outputs of the decentralized periphery station (ET200). On the other hand, pumps for the filling and harvesting processes are driven by means of a variable frequency drive (VFD), which is connected to the PLC using the same fieldbus (PROFINET).

2.4. Analytical Methods

The morphology of the microalgae species in the different media was analyzed using an optical microscope, Leica DM 500. Throughout the experimentation, at least three times a week, a sample of each culture was observed by microscope.

At the end of the experiment, after the exponential phase and spending various days (2–3 days) in the stationary state, the culture contained in the photobioreactor was harvested completely for its subsequent analysis. The steady state was determined by optical density using a colorimeter (Photometer D-100, Dinko Instruments, Baarcelona, Spain) in which a 580 nm filter was used. The optical density value for each sample was obtained as an average of three measurements. A straight line of calibration in which "Optical density" and "Concentration (mg/L)" were confronted, where the results obtained showed that for values between 0 and 1.5 absorbance units, linearity was obtained with a correlation coefficient r^2 of 0.9944. To obtain the dry biomass, previously proceeded to its centrifugation by means of laboratory centrifuge (Hettich ROTOFIX 32 A, Kirchlengern, Germany) with a velocity of 4000 rpm during 10–15 min, depending on centrifugated microalga, because some microalgae, for their morphology, size and excretion of gelatinous substances, required some minutes more to reach a good separation without to lose biomass. The next step was drying the biomass in a laboratory stove at 60–70 °C, and then the sample milling with electric mill model Super Junior "S" 180W Moulinex (908798).

Microalgae biomass characterization was performed by means of elemental analysis, calorific value, lipid and sugar contents.

The elemental analysis was carried out in an elemental analyzer (Leco CHNS True Spec 4084, St. Joseph, Michigan, USA) in accordance with UNE-EN 15104 standard norm for biomass C, H, N and S analysis.

The higher heating value (HHV) was determined by Parr 1351 calorimeter bomb (ISO 1928).

The carbohydrate content was determined using the modified quantitative saccharification method reported by the National Renewable Energy Laboratory (NREL), following the Moxley and Zhang method [41]. For this determination, high performance liquid chromatography (HPLC) LC-MS/MS Varian (310_MS TQ) was utilized using a Hiplex Na column 300 × 7.7 mm. This technique has been also used by Ho et al. [42], to evaluate the carbohydrate content of *Chorella vulgaris* biomass.

Lipid extraction was performed by Soxhlet extraction method with an organic solvent, utilizing hexane as a liquid solvent and using a fats extractor equipment (Det. GRAS N 2p 4002841, Abrera, Barcelona, Spain). The hexane solvent was evaluated as an extraction solvent for microalgae in the Soxhlet system with interesting results [43]. This is relatively inexpensive, easy to recover after extraction, and possesses selectivity to neutral lipids. Biomass was milled for a better homogenization and thus to favor the mechanical destruction of cells with the subsequent release of lipids. The sample was dried for 24 h at 105 °C in a laboratory stove. About 3 mg of sample was weighed into each cartridge, previously dried in an oven for at least 2 h at 105 °C, and then proceeded to the extraction of each sample until complete extraction. This determination was performed by triplicate to obtain the standard deviation. *"La Orden"* microalgae species oil was characterized by the measurement of the fatty acid profile; this method was described in previous works [44]. The anaerobic digestion of *"La Orden"* microalga biomass was carried out in 5 L stainless steel cylindrical reactors Batch LEHMANN Maschinenbau GMBH model built-in. Reactions were accomplished under a mesophilic regime for microorganisms, which implies that the

product contained in the digesters should be kept at 38 °C. Digesters had an automatic temperature and stirring control system. The retention time of the experiments was around 30 days. A daily sample with a 50 mL syringe was extracted on the top of the digester to determine different parameters and measure the biogas and methane production. The biogas was collected in an inverted test tube and was measured daily by the water displacement method. The biogas concentration was analyzed through a gases Sewerin Multitec 540 model analyzer. The following parameters: COD, TS, VS, pH, redox potential and alkalinity in the anaerobic digestion process were evaluated.

The medium or co-substrate pH and redox potential (E) measures were made by a pH-meter (Crison) connected to specific electrodes. The alkalinity, total solids (TS), and volatile solids (VS) were determined according to APHA, methods 2320 and 2540 E, respectively (APHA, 1998). Chemical oxygen demand (COD) was determined according to the standard EPA 410.4 [45]. The ratio C:N was obtained in the substrates using an elemental analyzer True-Spec CHN Leco 4084 model above mentioned.

2.5. Growth Kinetic

The quantification of biomass was determined daily through fiberglass filters of 0.45 µm pore diameter, measuring a daily volume of 25 mL by triplicate in laboratory photobioreactors and a 250 mL volume of sample in the pilot plant photobioreactors (column and flat plate). It was carried out by the dry weight method to constant weight (24–48 h) at 105 °C in an oven. Growth rate (μ_i) was determined using the expression [38]:

$$\mu_i = (Ln\ X_0 - Ln\ X_t)/(t_t - t_0)\ (\text{days}^{-1}) \quad (1)$$

μ_{max} considering, that is the highest growth rate during the exponential phase of the culture. The generation time (d_t) was determined with the equation [46]:

$$d_t = (Ln\ (2)/\mu_i)\ (\text{days}) \quad (2)$$

as well as daily biomass productivity (P_i), with the equation [38]:

$$P_i = (X_t - X_0)/(t_t - t_0)\ (\text{g L}^{-1}\ \text{d}^{-1}) \quad (3)$$

where X_0 is the initial biomass concentration (g L^{-1}) at time t_0 (d) and X_t biomass concentration (g L^{-1}) at any time t (d) subsequent to t_0. P_{max} is the largest considered productivities daily during the exponential phase of the culture.

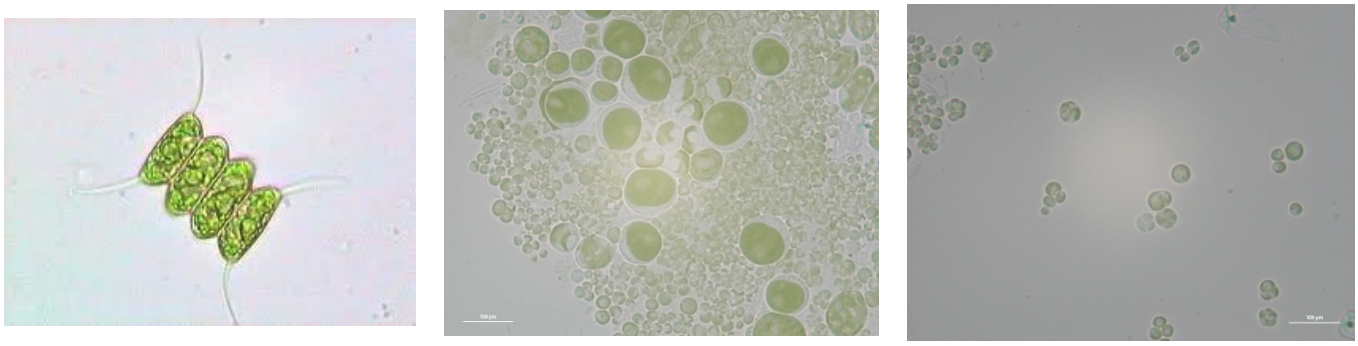
3. Results and Discussion

3.1. Microalgae Morphological Characterization

The monoalgae cultures observed have the following morphological characteristics:

- *Scenedesmus* sp.: it belongs to the Chlorophyta division, Chlorophyceae class, and to the *Scenedesmus* genus. It is characterized because it can form immobile colonies of lined cells forming flat sheets. The more frequent colonies have two or four cells but can have eight, sixteen, thirty-two and sometimes be unicellular. Normally, the end cells have two thorns up to 200 µm length that stand out. Each cell contains a unique parietal and one cellular wall (see Figure 3a).
- *Charca Brovales:* At the beginning, this population belonged to a green-blue cyanobacteria group and different types of microalgae; among them, different cells were observed, with various shapes such as filamentous, round, oval and other microorganisms such as protozoa and bacteria. Therefore, it can be considered a consortium of different species. After a time period of 15–20 days of culturing and cooling in the laboratory photobioreactor with high CO_2 concentrations, a proliferation of one specific microalga was observed. This was the mono-specific culture of a very small alga with a round form similar to *Chlorella minutissima* and was selected to form part of this study (see Figure 3b).

– *Arrocampo:* The cells collected in this reservoir are green and round, and are very similar to *Chlorellas sp.* and *Nannochloropsis sp.* This microalga is unicellular and does not form colonies. Morphologically, it is very similar to the consortium named *Charca Brovales* (Figure 3c).



(**a**) (**b**) (**c**)

Figure 3. Micrographs by optical microscopy of (**a**) *Scenedesmus sp.* to magnifications of 200 µm, (**b**) *Charca Brovales* consortium to magnifications of 100 µm, and (**c**) *Arrocampo* to magnifications of 100 µm.

As has been previously described, the "*La Orden*" consortium is a mixture of different microalgae collected at different reservoirs located in the Extremadura region (SW of Spain). The samples were collected, cooled, and cultured at a laboratory photobioreactor with high CO_2 concentrations. Among others, the microorganisms detected in this medium were predominantly population *Scenedesmus* sp. living with other unicellular species of round form, *Chlorella*. In Figure 4 the species found from microscopic observation under different magnifications are shown.

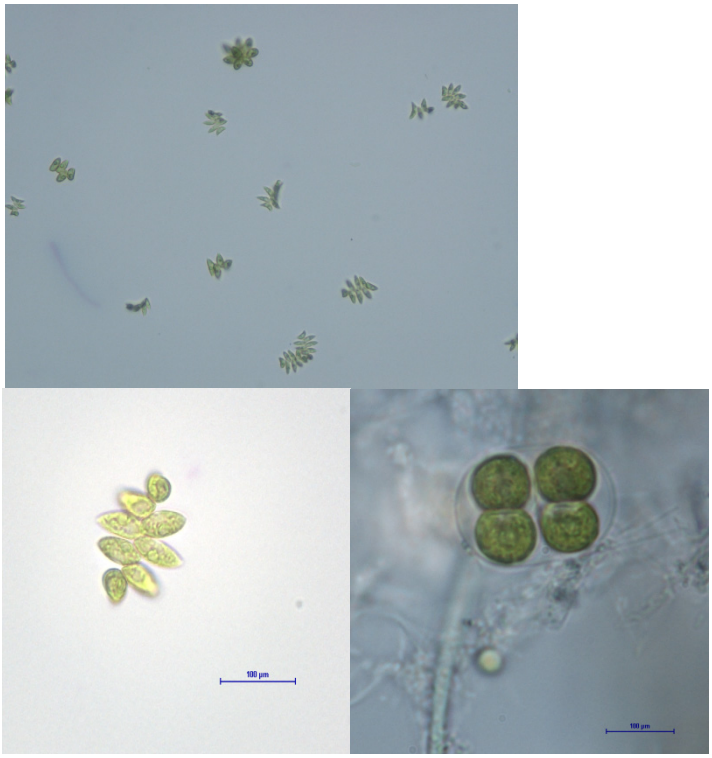


Figure 4. Micrographs by optical microscopy of "*La Orden*" consortium. Species found in the culture medium to magnifications of 20 µm (**above**), 100 µm (**left below**) and 100 µm (**right below**).

3.2. Microalgae Productivity and Growth Curves

3.2.1. Laboratory Photobioreactors

Growth curves obtained from the different microalgae populations in the different media were expressed as the biomass concentration versus time, see Figures 5 and 6. In the figures are given the mean values obtained from three samples of the biomass concentration oscillating with a deviation less than ±0.04. An exponential growth phase can be observed in every species, which extends along the first section of the high slope. In this phase, it is possible to determine the highest growth rates (μ_{max}), the lowest generation time (d_t) and the highest productivities (P_{max}). Table 2 collects the corresponding data to the two first parameters for this series. In Figure 5, one can see that the curve corresponding to *Scenedesmus* sp. is always above the *"La Orden"* consortium curve and this above *"Charca Brovales"* consortium one. However, the sequence for the μ_{max} value is *"La Orden" consortium* > *Scenedesmus* sp. > *"Charca Brovales" consortium* as is shown in Table 2. The sequence for the d_t value is *"La Orden" consortium* < *Scenedesmus* sp. < *"Charca Brovales" consortium*. For the fertilized medium, Figure 6 shows that the *Scenedesmus sp.* and *"La Orden"* consortium curves are practically overlapped at the beginning, indicating that μ_{max} and d_t are similar as can be seen in Table 2. In the case of the *"Charca Brovales" consortium*, the values of μ_{max} are higher and d_t are lower than for the other two species. The values given in Table 2 are lower than those reported by other authors, which have been carried out in pieces of research using autochthonous microalgae. For example, Martínez-García [47] obtained $\mu_{\max}$ of 1.75 d^{-1}, with d_t of 9.5 h for the *Synechocystis sp.* species. However, other authors [48] obtained values of d_t near 3 days in the best conditions for the *Nannochloropsis occulata* marine species. In addition, other published works report values of μ_{max} = 0.312 d^{-1} for *Chlorellas* [49], μ_{max} = 0.336 d^{-1} for the *Spirulina sp.* species [50] and μ_{max} = 1.09 d^{-1} for *Acutodesmus obliquus* [10].

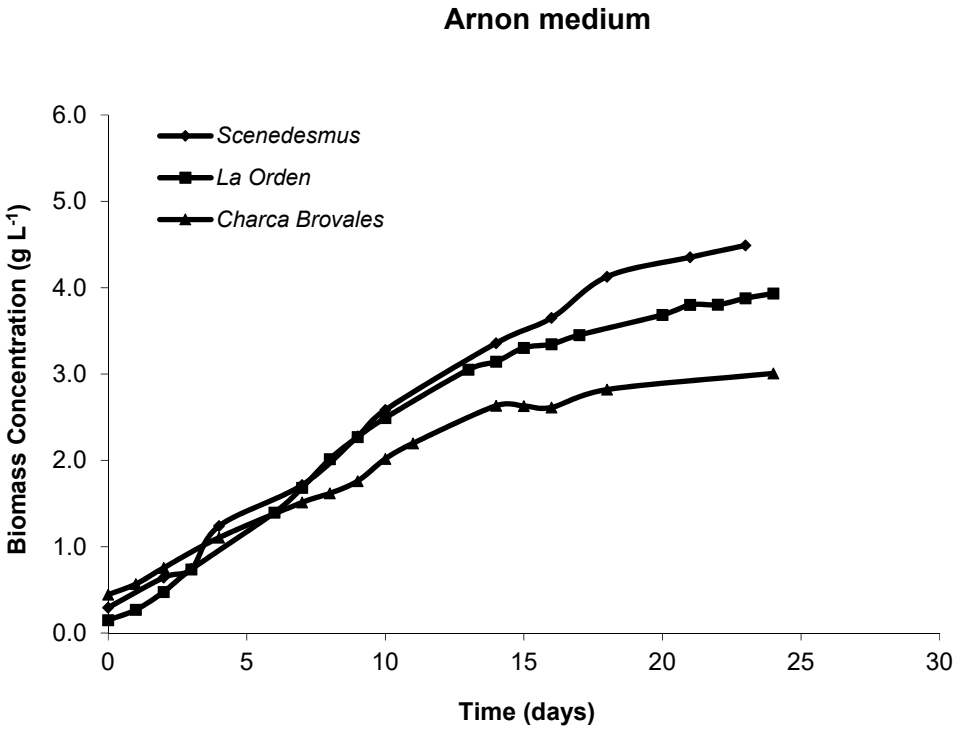


Figure 5. Growth curves of the different microalgae populations cultured in *Arnon* medium.

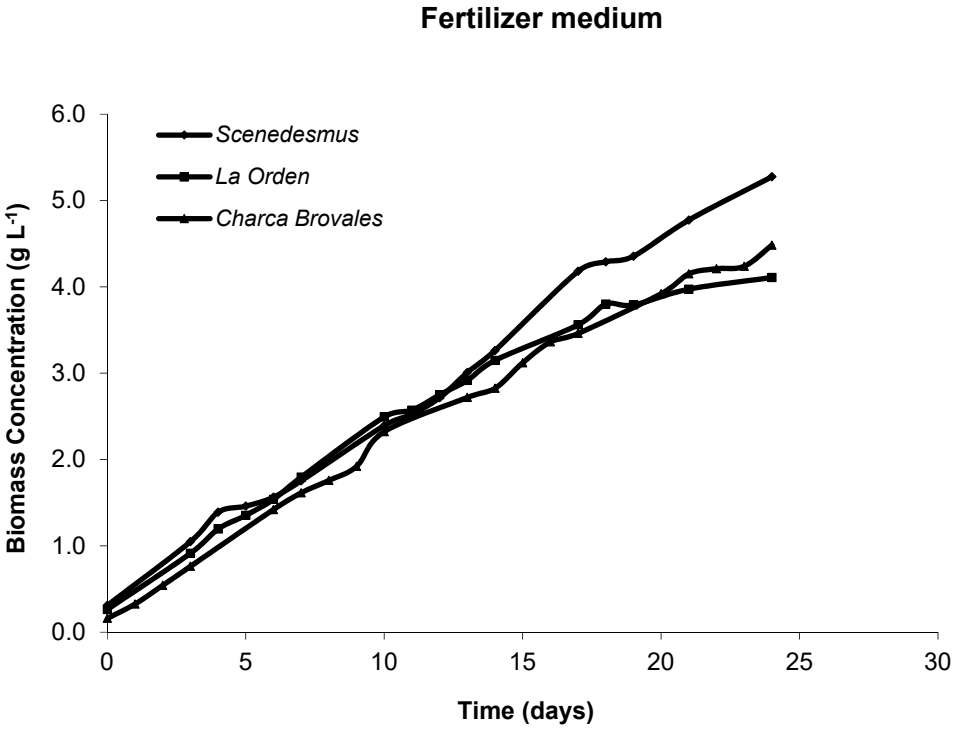


Figure 6. Growth curves of the different microalgae populations cultured in fertilizer medium.

Table 2. Highest maximum specific growth rates (μ_{max}) and lowest generation time (d_t) and maximum productivity (P_{max}).

Photobioreactor	Microalgae Population	μ_{max} (d $^{-1}$)	d_t (Days)	P_{max} (g L^{-1} d^{-1})
Laboratory	*Scenedesmus sp* (AM)	0.504	1.35	
	Scenedesmus sp (FM)	0.408	1.71	
	La Orden (AM)	0.864	0.81	
	La Orden (FM)	0.408	1.67	
	Charca Brovales (AM)	0.288	2.44	
	Charca Brovales (FM)	0.720	0.97	
Pilot Plant column	*La Orden* (FM)	0.360	2	0.116
Pilot plant panel	*La Orden* (FM)	0.312	2.2	0266

AM = Arnon medium; FM = fertilizer medium.

In this work, values of $\mu_{máx}$ and d_t of 0.504 d^{-1} and 1.35 days and 0.408 d^{-1} and 1.71 days were obtained for the *Scenedesmus sp.* species in AM and FM cultures, respectively. For this reference species, higher growth rates could be expected, since the bibliography report studies that, in similar conditions to the ones used in this work, have been obtained maximum specific growth rates of 1.128 d^{-1} and 1.056 d^{-1} [51,52]. Some authors as De Morais and Costa [50] have reported that some *Scenedesmus* species are fast photoautotrophic organisms, with maximum specific growth rates of 5.28 d^{-1} at optima conditions of culture. It is important to indicate that the maximum specific growth rates found in the bibliography correspond to the growth optimum conditions of each particular study. However, the general conditions of our study are taken in reference to the growth optima conditions of *Scenedesmus sp*. (reference microalga). This means that the specific rates of our tests could increase by studying the growth optima conditions of each autochthonous species analyzed. This will be reported in future works, where the light intensity in other seasons of the year and the ambient temperature between other variables will be analyzed.

Figure 7 shows the maximum productivity for the different microalgae populations for the two media assayed in this work. As it is shown, AM showed the best behavior for the *"Scenedesrmus sp."* and *"La Orden" consortium*, obtaining biomass maximum productivities of 0.498 and 0.336 g L^{-1} d^{-1}, respectively. However, the best results for the *"Charca Brovales"* species were obtained for the FM with a biomass maximum productivity of

0.402 g L^{-1} d^{-1}. It is important to indicate that the nitrogen concentrations of both media are different (double in AM media compared to FM media), which can lead to differences in biomass concentrations, affecting the behavior and growth of the different species. These results are representative if they are compared with other studies carried out with autochthon microalgae. For the reference species used in this work, however, higher growth rates were reached, with maximum productivities of 0.87–0.95 g L^{-1} d^{-1} [53]. In addition, very high maximum productivity values, such as 1.437 g L^{-1} d^{-1} for *Chlorella vulgaris* have been published [7]. Similar values to those obtained in this work have been published in the bibliography, such as 0.53–0.28, 0.37, 0.36, 0.48, 0.35–0.44 g L^{-1} d^{-1} for *Nannochloropsis gaditana, Tetraselmis chuii, Tetraselmis suecica and Phaeodactylum tricornutum* [54], *Chlorella vulgaris* [55], *Chlorella emersoni* [15], *Nannochloropsis sp.* [56] and *Scenedesmus ob.* [57], respectively. Also, the bibliography shows maximum productivity values lower than those obtained in this work, such as 0.15 g L^{-1} d^{-1} for *Scenedesmus ob.* [58] and 0.199 g L^{-1} d^{-1} [35] and 0.201 g L^{-1} d^{-1} [11] for *Chlorella vulgaris*, this last work to produce high-quality biofuel under culture stress conditions. In this study, the greatest amount of biomass accumulated during all the cultivation was obtained for the *Scenedesmus sp.* microalgae population with 5.66 g L^{-1}, followed by "*Charca Brovales*" with 5.52 g L^{-1}, both in the FM cultures. These productivities can be improved by studying the effect of light and temperature on growth and biomass composition. It will be carried out in future studies.

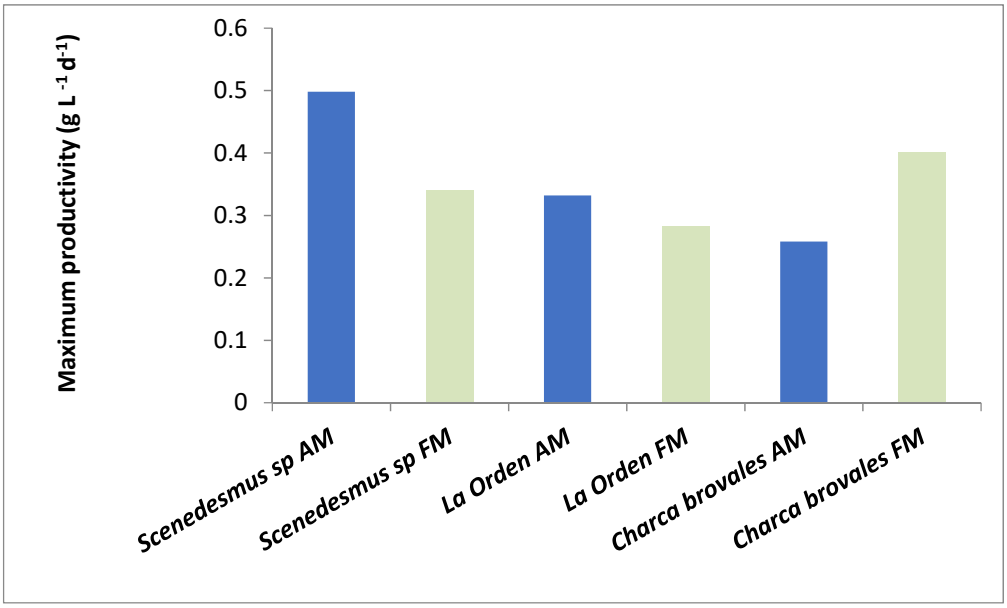


Figure 7. Maximum productivity for the different microalgae populations.

3.2.2. Pilot Plant

As has been described above, the growth of the *"La Orden"* consortium at two photobioreactors (column and flat panel) was studied in this plant using the FM culture. This culture was elected for this study, due to its better accessibility and lower cost than AM. This species was selected for this experimentation due to its better adaptation to the climatologic conditions of the zone, and besides, the consortium of different strains prevailed, it was verified in an analysis performed previously to this experimentation. Probably, the distribution of the different species varied, but to know this it would be necessary to carry out a species count. This will be performed in future studies. Figures 8 and 9 show the growth curves corresponding to *"La Orden"* species at column and flat panel photobioreactors, respectively, where the evolution of biomass concentration versus time is plotted. It can be observed that the increase of biomass concentration is practically linear with time, where light is most likely the limiting factor. They show low values in terms

of accumulated biomass and it may be due to the low temperatures (4–12 °C) and low brightness of January (high cloudiness) when these experiments were carried out.

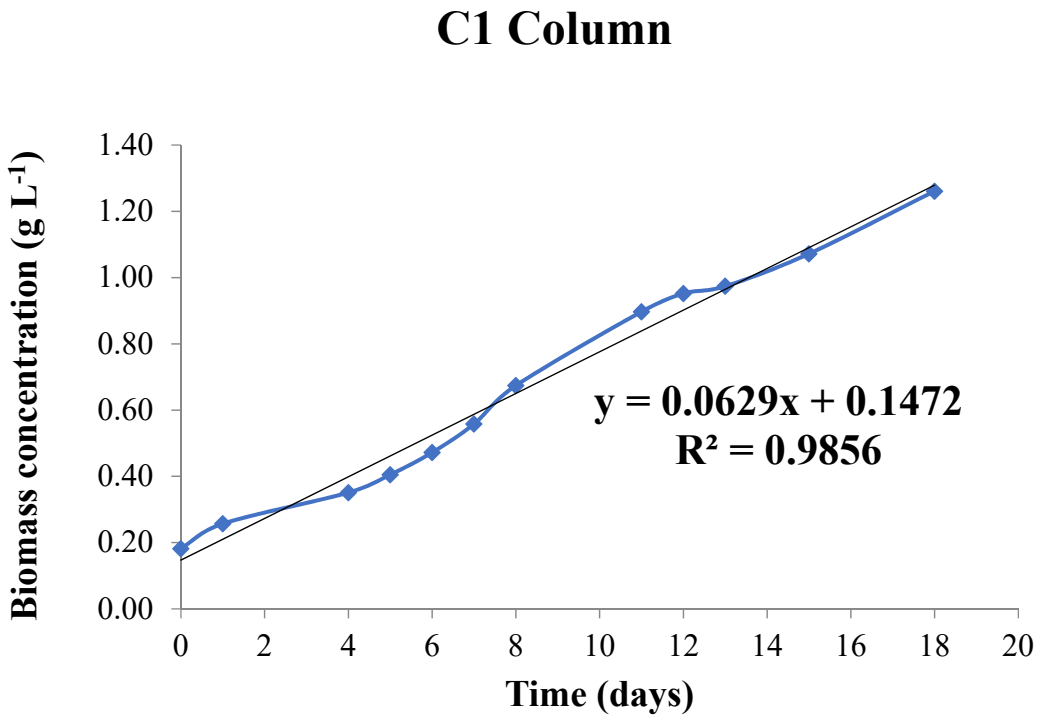


Figure 8. Growth curve of *"La Orden"* species with fertilizer medium in column photobioreactor of the pilot plant.

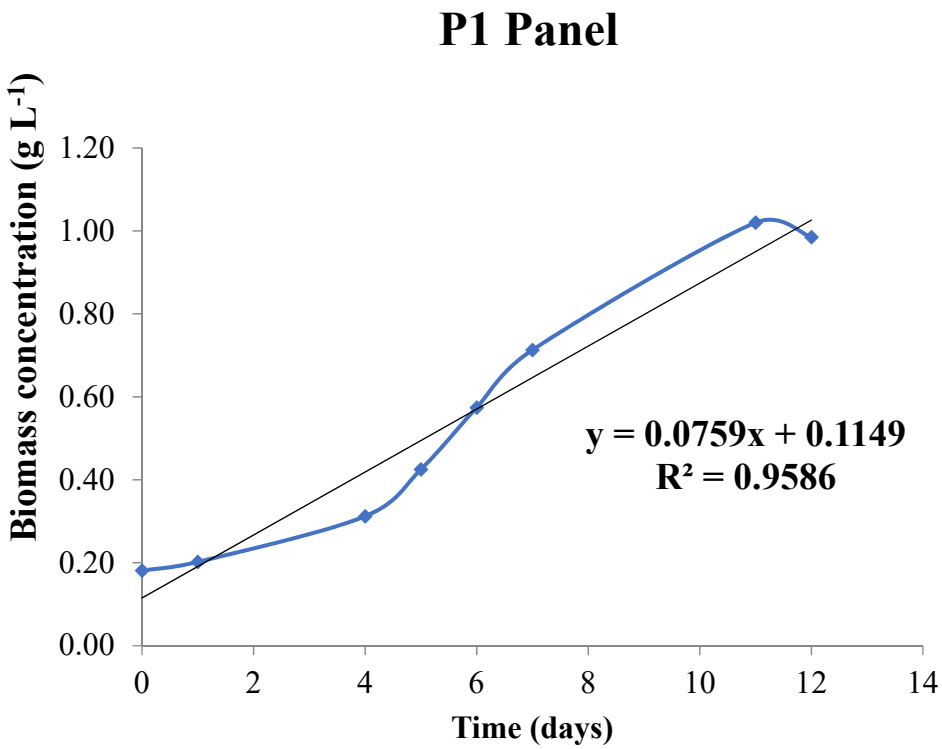


Figure 9. Growth curve of *"La Orden"* species with fertilizer medium in panel photobioreactor of the pilot plant.

Growth rates (μ_{max}) were also significantly lower than those expected and may be attributed to the lack of brightness and low temperatures of the period in which the study took place. This effect has been observed by other workers in this season (winter) since the availability of light is undoubtedly the main limiting factor for the photoautotrophs microalgae cultures [59]. Likewise, the temperature also influences the rate coefficients in the biosynthetic pathway of microalgae growth [60]. This parameter ranged between 6–15 °C during the study month. The unfavorable weather conditions and other factors such as high cell density, cells adhesion to the photobioreactor walls prevented the penetration of light into the culture. Thus, it was appreciated a slow exponential growth phase by light limitation, this fact has been also observed by other workers [38]. The results obtained in these experiments showed that the *"La Orden"* algae population has a similar growth rate and d_t in both systems, but the maximum productivity in the flat panel photobioreactor doubled to the obtained in the column photobioreactor (see Table 2). This effect may be attributable to the large illuminated surface of this type of photobioreactor, favoring the culture growth, as other workers have reported also [61].

3.3. Characterization of Microalgae Biomass from Laboratory Photobioreactors

In Table 3, the elemental analysis and HHV of the different microalgae populations are showed. There are no important differences in the C, H and S contents of the different species independently of the culture medium utilized, however, some differences are observed in the N content. While for "*Scenedesmus sp.*" and *"La Orden"* consortium species, the N content is higher for FM than AM, for the *"Charca Brovales"* consortium the N content is higher for AM. For this parameter, it is important to remember the different content of N for both culture media.

Table 3. Elemental analysis and HHV of the microalgae populations.

Microalgae Population	C/N	Elemental Analysis, % wt db				HHV, MJ kg^{-1}
		C	H	N	S	
Laboratory photobioreactor *Scenedesmus* sp. (AM)	17.9	43.0	6.65	2.79	0.356	21.2
Scenedesmus sp. (FM)	16.4	46.3	6.81	3.28	0.277	21.5
La Orden (AM)	16.5	48.9	7.28	3.46	0.245	21.8
La Orden (FM)	13.3	47.2	7.98	4.15	0.379	21.1
Charca Brovales (AM)	6.4	46.9	6.85	8.55	0.729	20.0
Charca Brovales (FM)	15.5	46.6	6.83	3.51	0.386	20.8
Exterior photobioreactors *La Orden* column (FM)	7.9	46.0	7.47	6.79	0.460	22.3
La Orden panel (FM)	6.7	48.3	6.76	8.41	0.592	20.7

Regarding the HHV of the microalgae biomass, one can see in Table 3 scant differences in this parameter in the different populations for the two culture media assayed in this work. The values obtained for the three species studied in this work are similar to those obtained by Scragg et al. [15], for *Chlorella vulgaris* and *Chlorella emersonii* in Watanabe medium. In addition, other authors [62] have published algae biomass HHV values of 24 MJ kg^{-1} (slightly higher than those obtained in this work) in a study on environmental life cycle comparison of algae and other bioenergy feedstocks, with the aim to evaluate their production to bio oil fuel, syngas and other products. Similar applications could be applied in our case, obtaining thermal or electric bioenergy (via combustion processes), biogas (via anaerobic digestion), syngas (via gasification process) to produce biofuels (via Fischer Tropsch reactions) or thermal and electric bioenergy (via internal combustion engines), and other high added value products.

The lipid contents extracted with hexane for the different microalgae populations are given in Table 4. The highest values of this parameter were obtained for AM in the populations *Scenedesmus sp* and *"La Orden"*. For the case of the *"Charca Brovales"* consortium, the highest value was obtained for FM. The main components of the microalgae biomass are proteins (30–60%), carbohydrates (20–30%), lipids (10–30%) and ashes (5–10%) [59]. Some extraction studies with hexane by Soxhlet method have determined a lipids percentage in dry biomass of the *Scenedesmus icrassatulus* and *Scenedesmus sp.* species of 8.2% and 7.02%, respectively [63]. The results obtained in this work are similar to those published by these authors and even higher for the species *"Scenedesmus sp."* and *"La Orden"* consortium, as can be observed in Table 4. Other authors have obtained lipids percentages higher than those obtained in this work, such as 56.6, 22.4, 12.3–17.5, 15.8% on a dry basis for *Chlorella vulgari* [34], *Scenedesmus obliquus* [57], *Acutodesmus obliquus* [10] and *Chlorella vulgari* [11], respectively.

Table 4. Lipids content of the microalgae populations extracted with hexane.

% wt db Lipids	
Microalgae Population	**Hexane**
Laboratory photobioreactor	
Scenedesmus sp. (AM)	9.3 ± 0.3
Scenedesmus sp. (FM)	8.8 ± 0.2
La Orden (AM)	12.6 ± 0.5
La Orden (FM)	8.8 ± 0.3
Charca Brovales (AM)	4.5 ± 0.4
Charca Brovales (FM)	7.4 ± 0.15
Exterior photobioreactors	
La Orden column (FM)	6.07 ± 0.03
La Orden panel (FM)	3.21 ± 0.19

Obviuosly, the production of biodiesel is considered as the most viable option to use these lipids contents. The results obtained by Chisti [22] highlight this enormous potential, with tests in pilot plants ongoing to prove the feasibility. However, plants at an industrial scale are still at early stages. Nevertheless, some of the pilot plants show considerable high productivities (of more than 100 g L^{-1} d^{-1}) biomass at least for a limited duration. However, the values given by Chisti [22], who found more than 100.000 L of biodiesel per ha are estimates based on the highest growth rates and highest oil contents.

The sugars content in the different populations for AM in laboratory photobioreactors was not analyzed due to the small accounts recollected of microalgae biomass in this reactors type. In the case of FM, in Table 5 are given the fructose, glucose and sucrose contents for the three species tested in this work. Although the values shown in Table 5 are low according to the total values mentioned by other authors, it can be observed that the highest value corresponds to glucose followed by fructose and sucrose for the three species studied. The production of bioethanol depends directly on the carbohydrates produced by algal biomass. Most of them are part of the cell wall structure and the composition remains variable for each microalgae species. Therefore, the conditions employed in the carbohydrates hydrolysis previous to the ethanol production by means of fermentation must be optimized for each microalgae. Thus, some researchers have reported values of 0.24, 0.52 and 0.40 g-ethanol/g-dry biomass for *Laminaria japonica* [29], *Chlorococcum sp.* [64] and *Chlorella vulgaris* [65], respectively.

Table 5. Sugars and carbohydrates content of the microalgae populations in fertilizer medium.

Microalgae Population	**Sugars Content % wt db**		
	Fructose	**Glucose**	**Sucrose**
Laboratory photobioreactor			
Scenedesmus sp	0.98	5.08	0.75
La Orden	0.71	1.45	0.51
Charca Brovales	0.81	2.01	0.31
Microalgae population	**Carbohydrates**		
	(% wt db)		
Exterior photobioreactors			
La Orden column		6.50	
La Orden panel		9.22	

3.4. Microalgae Biomass Characterization from Exterior Photobioreactors

In this section, the results obtained in panel and column photobioreactors are discussed with the aim of increasing the microalgae biomass production. The culture medium used was FM and the cultivated species was "*La Orden*". Tables 3 and 5 show, respectively, the elemental and HHV analysis and carbohydrates content, of the biomasses collected from the flat panel and column photobioreactors. Small differences in the elemental analysis and HHV of the biomass extracted from the two photobioreactors can be observed. Thus, the biomass extracted from the column has an H content and HHV higher than the extracted from the flat plate photobioreactor. However, the carbohydrates content of the biomass extracted from the flat panel is higher than the extracted one from the column photobioreactor. The carbohydrates content obtained in this work for "*La Orden*" microalga biomass is low compared to that obtained by other workers, as has been already commented above. This fact may be caused by a lack of nutrients; according to different authors, a nitrogen deficit causes a high accumulation of carbohydrates [66]. The N content analyzed in the biomass extracted from the two photobioreactors is high, as can be observed in Table 3 (values of 6.79% and 8.41% in elemental analysis), therefore it could be related to the lower content of carbohydrates.

On the other hand, the content of accumulated lipids in "*La Orden*" consortium biomass in the two exterior photobiorreactors was low, even lower than in the laboratory photobioreactor. However, the extracted biomass from the last one the N content obtained in the elemental analysis was also quite lower (see Table 3). This fact may be also due to the lack of nitrogen, in coherence with recent studies [25,66], which have shown that microalgae culture in nitrogen deficient conditions leads to an increase in the lipid or carbohydrate content, since there is a competition between the synthesis of lipids and sugars. However, the increase in lipids or carbohydrate contents under nitrogen stress conditions appears to differ depending on the strain [66]. Thus, in this work, the same reason is applied, suggesting that the cultivation of the column suffered no nutritional stress, and in consequence, it presented a lipids content higher than the culture carried out in the flat panel photobioreactor (see Table 4). Probably, the competition between the synthesis of lipids and sugars gave rise to an increase in the percentage of carbohydrates, reducing thus the percentage of lipids.

Table 6 collects the fatty acids composition of microalgae and vegetable oils to establish a comparison. The "*La Orden*" species oil has a major fatty acid, palmitic acid, which is a saturated fatty acid that gives the oil a high saturation rank, close to the 40% that is typical of animal fats (see Table 6) [67]. On the other hand, this oil contains a high number of not identified fatty acids that although in low concentrations, as can be observed in the chromatogram of Figure 10, are involved in the important total content. Also, the high oleic and linoleic acid contents are highlighted, as is expected in typical vegetable oils (see Table 6) where their contents are higher than in animal fats [27]. Comparing

the fatty acid composition of the five microalgae species oils included in Table 6, one can observe that *"La Orden"* species oil is a mix between *Scenedesmus ob.*, *Chlorella vulgaris* and *Acutodesmus obliquus* oils, since the distribution of *Parietochloris incisa* oil is very different to "*La Orden*". In the case of *"La Orden"* species oil utilization for biodiesel production, contrary effects were observed. On the one hand, the high saturation rank of this oil conferred poor properties at low temperatures, while the polyunsaturated fatty acids presence would improve these properties regarding the results obtained with animal fats biodiesel. Dissimilarly, the oxidation stability of the generated biodiesels would be favored by the presence of saturated fatty acids, while fatty acids such as linoleic and oleic would be more affected in the oxidation process [68].

Table 6. Comparison of the major fatty acid composition of microalgae with other feedstocks published by other workers ([10]: Yun et al., 2016; [11]: El-Sheekh et al., 2019; [25]: Bigogno et al., 2002; [27] Martínez et al., 2014; [57]: Ho et al., 2012; [67]: Encinar et al., 2011).

Origin	Animal Fats [67]	Rape Oil [27]	Soybean Oil [27]	*Scenedesmus ob.* Oil [57]	*Parietochloris incisa* Oil [25]	*Chlorella vulgaris* Oil [11]	*Acutodesmus obliquus* Oil [10]	*"La Orden"* Oil (this work)
Lauric acid C12:0						0.46 ± 0.05		
Myristic acid C14:0	1.38	0.07	0.09			1.33 ± 0.75		
Palmitic acid C16:0	27.3	4.92	11.60	19.80	10.00	34.37 ± 1.56	34.0	31.50 ± 1.37
Palmitoleic acid C16:1	4.01	0.24	0.11	4.06	2.00			2.41 ± 0.09
Stearic acid C18:0	11.7	1.63	3.25	9.08	3.00	4.75 ± 1.2		3.17 ± 0.09
Oleic acid C18:1	44.4	66.59	25.09	16.41	16.00	44.91 ± 2.65	13.0	19.60 ± 1.56
Linoleic acid C18:2	10.4	17.08	52.93	21.50	17.00	12.78 ± 1.87	7.8	9.32 ± 1.13
Linolenic acid C18:3	0.62	7.75	5.95	12.3	3.00	1.40 ± 0.05	36.0	6.25 ± 1.05
Nonadecanoic acid C19:0								4.71 ± 1.01
Arachidonic acid C20:4					43.00			
Others	0.19	1.71	0.99	16.60	3		9.2	23.10 ± 0.93

Studies on the biogas generation by anaerobic digestion from microalgae have been conducted since the 1950s [7]. In this work, the anaerobic digestion of *"La Orden"* biomass species collected from flat panel and column photobioreactors was made. The microalgae biomass introduced in the digester was fresh, as it was extracted from photobioreactors. The microalgae biomass digestion process was facilitated by introducing in the digester an inoculum, the ratio microalgae/inoculum used in this test was 2:1. In Table 7 the characteristics of the two substrates are given. The digestion process was carried out in a batch regime. The aim was simply to know the potential of CH_4 yield of the species cultured in the higher capacity photobioreactors. The CH_4 yield obtained was 296 ± 23 L CH_4 $kg_{VSS}{}^{-1}$ added with a reduction percentage of COD and vs. of 50 ± 1% and 50 ± 1.7%, respectively. Ras et al. [7] studied the anaerobic digestion of *Chlorella vulgaris* under 28 days hydraulic retention time with an organic loading rate of 1 g_{COD} L^{-1}, obtaining a methane production of 240 L $kg_{VSS}{}^{-1}$, they achieved a 51% COD removal. Debowski et al. [36] have obtained 351.88 L $kg_{VSS}{}^{-1}$ in the anaerobic codigestion of perennial crop *Sida hermaphrodita* and microalgae biomass under semi-continuous conditions. Wang and Park [9] obtained a higher CH_4 yield on the volatile solids fed to the digester for *Chlorella sp.* (230 L $kg_{VSS}{}^{-1}$) than *Micractinium sp.* (209 L $kg_{VSS}{}^{-1}$).

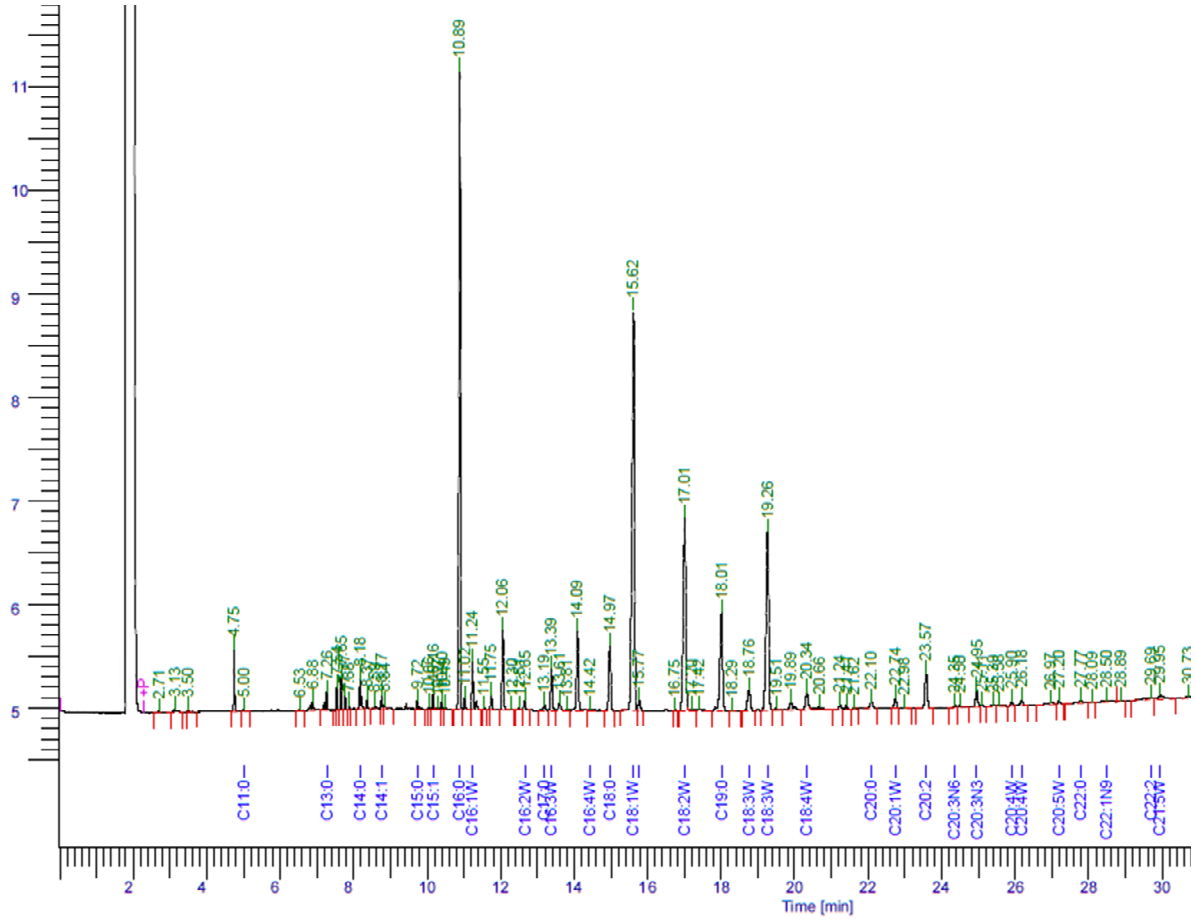


Figure 10. Chromatogram of fatty acid profile from *"La Orden"* microalga oil.

Table 7. Initial characterization of substrates used in the anaerobic digestion process.

Parameter	pH	E, mV	TS, %	VS, %	VS_T, %	COD_T, mg O_2 L^{-1}	N-ammonia, mg L^{-1}	C:N	Alkalinity, mg $CaCO_3$ L^{-1}
"La Orden" consortium	7.24	53	9.80	65.00	6.37	150,000	196	7.55	3754
Inoculum	7.50	−414	2.86	51.29	1.47	47,000	1740	9.13	8568

COD_T: Total chemical oxygen demand, mg O_2 L^{-1}. VS_T: Volatile solid over total solid, %.

4. Conclusions

In this work, a study of different microalgae species for biomass production, as well as the possibilities to obtain biorefinery products has been carried out. Growth curves of three autochthonous microalgae in laboratory photobioreactors in AM and FM culture media were studied. The maximum growth rates, minimum generation times and maximum productivities were analyzed.

The best results for both media were obtained in *Scenedesmus sp.* and *"Charca Brovales"* consortium, respectively.

The elemental analysis and HHV of biomass for all species were independent of the culture medium used.

The lipids highest values were obtained for Arnon medium in *Scenedesmus sp.* and *"La Orden"* consortium and for agricultural liquid fertilizers medium in *"Charca Brovales"* consortium.

The sequence of sugars value was glucose > fructose > sucrose in all the species for FM.

The study was also performed in pilot plant (column and flat panel) photobioreactors for the *"La Orden"* consortium operated by an automation and monitoring system. The maximum

growth rates and maximum productivities were lower in these photobioreactors than in the laboratory one. In addition, some differences were obtained for lipids and sugars contents. The fatty acids profile was obtained for *"La Orden"* consortium oil and its composition is between animal fat and vegetable oil one. Also, the CH_4 yield in a batch type digestion process of this species was evaluated, obtaining a value of 296 ± 23 L CH_4 kg_{VSS}^{-1} added with a reduction percentage of COD and vs. of 50±1% and 50 ± 1.7%, respectively.

In future research, our group will try to subject the microalgae strains to different stress conditions in order to increase the production of carbohydrates and lipids for their transformation into biofuels, as well as incorporating water from reservoirs into the photobioreactors, and thus evaluating the effect of real conditions.

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References

1. European Commission. 2030 Objectives. Available online: http://ec.europa.eu/europe2020/index_es.htm (accessed on 20 February 2021).
2. Suwelack, K.; Wüst, D. An approach to unify the appraisal framework for biomass conversion systems. *Biomass Bioenergy* **2015**, *83*, 354–365. [CrossRef]
3. Okoro, V.O.; Sun, Z.; Birch, J. Meat processing waste as a potential feedstock for biochemicals and biofuels- A review of possible conversion technologies. *J. Clean. Prod.* **2017**, *142*, 1583–1608. [CrossRef]
4. Dayananda, C.; Sarada, R.; Rani, M.U.; Shamala, T.R.; Ravishankar, G.A. Autotrophic cultivation of *Botryococcus braunii* for the production of hydrocarbons and exopolysaccharides in various media. *Biomass Bioenergy* **2007**, *31*, 87–93. [CrossRef]
5. Deprá, M.C.; Dos Santos, A.M.; Severo, I.A.; Santos, A.B.; Zepka, L.Q.; Jacob-Lopes, E. Microalgal Biorefineries for Bioenergy Production: Can We Move from Concept to Industrial Reality? *BioEnergy Res.* **2018**, *11*, 727–747. [CrossRef]
6. Pienkos, P.; Darzins, A. The promise and challenges of microalgal-derived biofuels. *Biofuel Bioprod. Biorefining* **2009**, *3*, 431–440. [CrossRef]
7. Ras, M.; Lardon, L.; Sialve, B.; Bernet, N.; Steyer, J.P. Experimental study on a coupled process of production and anaerobic digestion of *Chorella vulgaris*. *Bioresour. Technol.* **2011**, *102*, 200–206. [CrossRef] [PubMed]
8. Debowski, M.; Zielinski, M.; Grala, A.; Dudek, M. Algae biomass as an alternative substrate in biogas production technologies—Review. *Renew. Sustain. Energy Rev.* **2013**, *27*, 596–604. [CrossRef]
9. Wang, M.; Park, C. Investigation of anaerobic digestion of *Chlorella sp.* and *Micractinium sp.* grown in high-nitrogen wastewater and their co-digestion with waste activated sludge. *Biomass Bioenergy* **2015**, *80*, 30–37. [CrossRef]
10. Yun, H.S.; Ji, M.K.; Park, Y.T.; Salama, E.S.; Choi, J. Microalga, *Acutodesmus obliquus* KGE 30 as a potential candidate for CO_2 mitigation and biodiesel production. *Environ. Sci. Pollut. Res.* **2016**, *23*, 17831–17839. [CrossRef]
11. El-Sheekh, M.M.; Gheda, S.F.; El-Sayed, A.E.B.; Shady, A.M.A.; El-Sheikh, M.E.; Schagerl, M. Outdoor cultivation of the green microalga *Chlorella vulgaris* under stress conditions as a feedstock for biofuel. *Environ. Sci. Pollut. Res.* **2019**, *26*, 18520–18532. [CrossRef]
12. Batan, L.; Quinn, J.; Willson, B.; Bradley, T. Net energy and greenhouse gas emission evaluation of biodiesel derived from microalgae. *Environ. Sci. Technol.* **2010**, *44*, 7975–7980. [CrossRef]

13. Liu, X.; Saydah, B.; Eranki, P.; Colosi, L.M.; Mitchell, B.G.; Rhodes, J.; Clarens, A.F. Pilot-scale data provide enhanced estimates of the life cycle energy and emissions profile of algae biofuels produced via hydrothermal liquefaction. *Bioresour. Technol.* **2013**, *148*, 163–171. [CrossRef] [PubMed]
14. Moody, J.W.; McGinty, C.M.; Quinn, J.C. Global evaluation of biofuel potential from microalgae. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 8691–8696. [CrossRef] [PubMed]
15. Scragg, A.H.; Illman, A.M.; Carden, A.; Shales, S.W. Growth of microalgae with increased calorific values in a tubular bioreactor. *Biomass Bioenergy* **2002**, *23*, 67–73. [CrossRef]
16. Wijffels, R.; Barbosa, M. An Outlook on Microalgal Biofuels. *Science* **2010**, *329*, 796–799. [CrossRef]
17. Norsker, N.H.; Barbosa, M.J.; Vermue, M.H.; Wijffels, R.H. Microalgal production—A close look at the economics. *Biotechnol. Adv.* **2011**, *29*, 24–27. [CrossRef]
18. Murphy, C.F.; Allen, D.T. Energy-water nexus for mass cultivation of algae. *Environ. Sci. Technol.* **2011**, *45*, 5861–5868. [CrossRef]
19. Alba, L.; Torri, C.; Samorì, C.; van der Spek, J.; Fabbri, D.; Kersten, S.R.A.; Brilman, D.W.F. Hydrothermal treatment (HTT) of microalgae: Evaluation of the process as conversion method in an algae biorefinery concept. *Energy Fuels* **2012**, *26*, 642–657. [CrossRef]
20. Mu, D.; Min, M.; Krohn, B.; Mullins, K.A.; Ruan, R.; Hill, J. Life cycle environmental impacts of wastewater-based algal biofuels. *Environ. Sci. Technol.* **2014**, *48*, 11696–11704. [CrossRef] [PubMed]
21. Mu, D.; Ruan, R.; Addy, M.; Mack, S.; Chen, P.; Zhou, Y. Life cycle assessment and nutrient analysis of various processing pathways in algal biofuel production. *Bioresour. Technol.* **2017**, *230*, 33–42. [CrossRef]
22. Chisti, Y. Biodiesel from microalgae. *Biotechnol. Adv.* **2007**, *25*, 294–306. [CrossRef] [PubMed]
23. Suali, E.; Sarbatly, R. Conversion of microalgae to biofuel. *Renew. Sustain. Energy Rev.* **2012**, *16*, 4316–4342. [CrossRef]
24. Kunjapur, A.M.; Bruce-Eldridge, R. Photobioreactor Design for Commercial Biofuel Production from Microalgae. *Ind. Eng. Chem. Res.* **2010**, *49*, 3516–3526. [CrossRef]
25. Bigogno, C.; Khozin-Goldberg, I.; Boussiba, S.; Vonshaka, A.; Cohen, Z. Lipid and fatty acid composition of the green oleaginous alga *Parietochloris incisa*, the richest plant source of arachidonic acid. *Phytochemistry* **2002**, *60*, 497–503. [CrossRef]
26. Encinar, J.M.; González, J.F.; Sabio, E.; Ramiro, M.J. Preparation and properties of biodiesel from *Cynara cardúnculus L.* oil. *Ind. Eng. Chem. Res.* **1999**, *38*, 2927–2931. [CrossRef]
27. Martínez, G.; Sánchez, N.; Encinar, J.M.; González, J.F. Fuel properties of biodiesel from vegetable oils and oil mixtures. Influence of methyl esters distribution. *Biomass Bioenergy* **2014**, *63*, 22–32. [CrossRef]
28. Sánchez, N.; Sánchez, R.; Encinar, J.M.; González, J.F.; Martínez, G. Complete analysis of castor oil methanolysis to obtain biodiesel. *Fuel* **2015**, *147*, 95–99. [CrossRef]
29. Kim, N.J.; Li, H.; Jung, K.; Chang, C.H.; Lee, P.C. Ethanol production from marine algal hydrolysates using *Escherichia coli* KO11. *Bioresour. Technol.* **2011**, *102*, 7466–7469. [CrossRef]
30. John, R.P.; Anisha, G.S.; Nampoothiri, K.M.; Pandey, A. Micro and macroalgal biomass: A renewable source for bioethanol. *Bioresour. Technol.* **2011**, *102*, 186–193. [CrossRef] [PubMed]
31. Matías, J.; Encinar, J.M.; González, J.; González, J.F. Optimisation of ethanol fermentation of Jerusalem artichoke tuber juice using simple technology for a decentralised and sustainable ethanol production. *Energy Sustain. Dev.* **2015**, *25*, 4–39. [CrossRef]
32. Nahak, S.; Nahak, G.; Pradhan, I.; Sahu, R.K. Bioethanol from Marine Algae: A Solution to Global Warming Problem. *J. Appl. Environ. Biol. Sci.* **2011**, *1*, 74–80.
33. Branyikova, I.; Marsalkova, B.; Doucha, J.; Branyik, T.; Bisova, K.; Zachleder, V. Microalgae-novel highly efficient starch producers. *Biotechnol. Bioeng.* **2011**, *108*, 766–776. [CrossRef] [PubMed]
34. Liu, Z.Y.; Wang, G.C.; Zhou, B.C. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. *Bioresour. Technol.* **2008**, *99*, 4717–4722. [CrossRef] [PubMed]
35. Dragone, G.; Fernandes, B.D.; Abreu, A.P.; Vicente, A.A.; Teixeira, J.A. Nutrient limitation as a strategy for increasing starch accumulation in microalgae. *Appl. Energy* **2011**, *88*, 3331–3335. [CrossRef]
36. Dębowski, M.; Zieliński, M.; Kisielewska, M.; Krzemieniewski, M. Anaerobic Co-digestion of the Energy Crop *Sida hermaphrodita* and Microalgae Biomass for Enhanced Biogas Production. *Int. J. Environ. Res.* **2017**, *11*, 243–250. [CrossRef]
37. Arnon Daniel, I.; McSwain, B.D.; Tsujimoto, H.Y.; Wada, K. Photochimical activity and components of membrane preparations from blue-green algae I. Coexistence of two photosystems in relation to cholorphyll and removal of phycoyanin. *Biochim. Biophys. Acta* **1974**, *357*, 231–245. [CrossRef]
38. Andersen, R.A. *Algal Culturing Techniques*; Elsevier Academic Press: Burlington, MA, USA, 2005.
39. Chisti, Y. Production of microalgae for energy—An introduction. In Proceedings of the International Technical Conference on "Production and Use of Microalgae for Energy Purposes", Madrid, Spain, 11 November 2008.
40. Calderón, A.J.; González, I. Some hardware and instrumentation aspects of the development of an automation system for jar tests in drinking water treatment. *Sensors* **2017**, *17*, 2305. [CrossRef]
41. Moxley, G.; Zhang, Y.H.P. More accurate determination of acid-labile carbohydrates in lignocellulose by modified quantitative saccharification. *Energy Fuels* **2007**, *21*, 684–3688. [CrossRef]
42. Ho, S.H.; Huang, S.W.; Chen, C.Y.; Hasunuma, T.; Kondo, A.; Chang, J.S. Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. *Bioresour. Technol.* **2013**, *135*, 191–198. [CrossRef]
43. Anderson, J.; Sorek, B. *Microalgae: The Fuel of Tomorrow*; University of Pittsburg: Pittsburgh, PA, USA, 2008.

44. Encinar, J.M.; González, J.F.; Martínez, G.; Sánchez, N.; Pardal, A. Soybean oil transesterification by the use of a microwave flow system. *Fuel* **2012**, *95*, 386–393. [CrossRef]
45. EPA 414.4. *Methods for Chemical Analysis of Water and Wastes*; U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory: Washington, DC, USA, 1993.
46. Bailey, J.E.; Ollis, D.F. *Biochemical Engineering Fundamentals*, 2nd ed.; McGraw-Hill: Singapore, 1986.
47. Martínez-García, L. Elimination of CO_2 with Native Microalgae. Ph.D. Thesis, University of León, Institute of Natural Resources, Area of Chemical Engineering, León, Spain, 2009; p. 213.
48. Persico, M.M.; Moris, M.; Tranier, E.D.; Zanazzi, A.N.; Saubidet, A.A.; Beligni, M.V. Evaluación de un sistema exterior de cultivo masivo de la microalga marina *Nannochloropsis occulata*, en una zona templada oceánica de Argentina. *Revista Latinoamericana de Biotecnología Ambiental y Algal* **2011**, *2*, 30–48.
49. Chang, E.H.; Yang, S.S. Some characteristics of microalgae isolated in Taiwan for biofixation of carbon dioxide. *Bot. Bull. Acad. Sin.* **2003**, *44*, 43–52.
50. De Morais, M.G.; Costa, J.A. Biofixation of carbon dioxide by *Spirulina* sp. and *Scenedesmus obliquus* cultivated in a three-stage serial tubular photobioreactor. *J. Biotechnol.* **2007**, *129*, 439–445. [CrossRef] [PubMed]
51. Martínez, M.E.; Jiménez, J.M.; Farida, E.Y. Influence of phosphorus concentration on the growth kinetics and stoichiometry of the microalga *Scenedesmus obliquus*. *Process. Biochem.* **1997**, *32*, 657–664. [CrossRef]
52. Hodaifa, G.; Martínez, M.; Sánchez, S. Use of industrial wastewater from olive-oil extraction for biomass production of *Scenedesmus obliquus*. *Bioresour. Technol.* **2008**, *99*, 1111–1117. [CrossRef] [PubMed]
53. Sánchez, J.F.; Fernández-Sevilla, J.M.; Acién, F.G.; Cerón, M.C.; Pérez-Parra, J.; Molina-Grima, E. Biomass and lutein productivity of *Scenedesmus almeriensis*: Influence of irradiance, dilution rate and temperature. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 719–729. [CrossRef] [PubMed]
54. San Pedro, A.; González-López, C.V.; Acién, F.G.; Molina-Grima, E. Marine microalgae selection and culture conditions optimization for biodiesel production. *Bioresour. Technol.* **2013**, *134*, 353–361. [CrossRef]
55. Illman, A.M.; Scragg, A.H.; Shales, S.W. Increase in chlorella strains calorific values when grown in low nitrogen medium. *Enzym. Microb. Technol.* **2000**, *27*, 631–635. [CrossRef]
56. Chiu, S.Y.; Kao, C.Y.; Tsai, M.T.; Ong, S.C.; Chen, C.H.; Liu, C.S. Lipid accumulation and CO_2 utilization of *Nannochloropsis occulata* in response to CO_2 aeration. *Bioresour. Technol.* **2009**, *100*, 833–838. [CrossRef]
57. Ho, S.H.; Chen, C.Y.; Chang, J.S. Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. *Bioresour. Technol.* **2012**, *113*, 244–252. [CrossRef] [PubMed]
58. Mandal, S.; Mallick, N. *Scenedesmus obliquus* as a potential source of biodiesel production. *Appl. Microbiol. Biotechnol.* **2009**, *84*, 281–291. [CrossRef] [PubMed]
59. Molina-Grima, E.; Fernández-Sevilla, J.M.; Sánchez-Pérez, J.A.; García-Camacho, F. A study on simultaneous photolimitation and photoinhibition in dense microalgal cultures taking into account incident and averaged irradiances. *J. Biotechnol.* **1996**, *45*, 59–69. [CrossRef]
60. Richmond, A. Microalgaculture. *Crit. Rev. Biotechnol.* **1986**, *4*, 369–438. [CrossRef]
61. Tredici, M.R.; Chini Zitelli, G. Efficiency of sunlight utilization: Tubular versus flat fotobioreactors. *Biotechnol. Bioeng.* **1998**, *57*, 187–197. [CrossRef]
62. Clarens, A.F.; Resurreccion, E.P.; White, M.A.; Colosi, L.M. Environmental life cycle comparison of algae to other bioenergy feedstocks. *Environ. Sci. Technol.* **2010**, *44*, 1813–1819. [CrossRef]
63. Punín-Crespo, M.O.; Lage-Yusty, M.A. Determination of aliphatic hydrocarbons in the alga *Himanthalia elongate*. *Ecotoxicol. Environ. Saf.* **2004**, *57*, 226–230. [CrossRef] [PubMed]
64. Harun, R.; Danquah, M.K.; Forde, G.M. Microalgal biomass as a fermentation feedstock for bioethanol production. *J. Chem. Technol. Biotechnol.* **2010**, *85*, 199–203. [CrossRef]
65. Lee, S.; Oh, Y.; Kim, D.; Kwon, D.; Lee, C.; Lee, J. Converting carbohydrates extracted from marine algae into ethanol using various ethanolic *Escherichia coli* strains. *Appl. Microbiol. Biotechnol.* **2011**, *164*, 878–888. [CrossRef]
66. Ho, S.H.; Huang, S.W.; Chen, C.Y.; Hasunuma, T.; Kondo, A.; Chang, J.S. Characterization and optimization of carbohydrate production from an indigenous microalgae *Chorella vulgaris* FSP-E. *Bioresour. Technol.* **2013**, *135*, 157–165. [CrossRef]
67. Encinar, J.M.; Sánchez, N.; Martínez, G.; García, L. Study of biodiesel production from animal fats with high free fatty acid content. *Bioresour. Technol.* **2011**, *102*, 10907–10914. [CrossRef]
68. Sánchez, N. Obtención de Biodiésel Mediante Transesterificación de Aceite de Ricino y Grasas Animales. Aprovechamiento Energético de la Glicerina Como Subproducto Del Proceso. Ph.D. Thesis, Universidad de Extremadura, Badajoz, Spain, 18 May 2015.