

Article

Seasonal Influence on the Yield and Chemical Composition of Biofuels Derived from Raceway-Grown Microalgae

Ailin Rasaie ¹, Indira Tobío ^{1,2}, David Bolonio ^{1,*}, Blanca Castells ¹, Magín Lapuerta ² and Laureano Canoira ¹

¹ Department of Energy & Fuels-Tecminergy, ETS Ingeniería de Minas y Energía, Universidad Politécnica de Madrid, Ríos Rosas 21, 28003 Madrid, Spain; laureano.canoira.lopez@upm.es (L.C.)

² Grupo de Combustibles y Motores, ETS Ingeniería Industrial, Universidad de Castilla-La Mancha, Avda. Camilo José Cela s/n, 13071 Ciudad Real, Spain; magin.lapuerta@uclm.es

* Correspondence: david.bolonio@upm.es

Abstract

This study evaluates the influence of seasonal harvesting conditions on biomass productivity, oil yield, and biodiesel-related FAME composition of native microalgae—predominantly *Chlorella* sp., with minor populations of *Scenedesmus* and *Ankistrodesmus*—cultivated in an open raceway pond integrated into a pig-slurry biogas plant in Soria, Spain. Processing biomass harvested across four seasons showed that oven drying at 45 °C yields stable seasonal biomass concentrations around 7.80–8.80 g/L, while extended outdoor air-drying during spring and summer reduces yields to 2.86 and 4.33 g/L due to the absence of active growth conditions. Lipids extracted via the Soxhlet method using a dichloromethane: methanol mixture (9:1 v/v) exhibited a wide range of 2.5–18.9 g oil per 100 g dry biomass. Marked seasonal variations were observed: winter samples demonstrated an outstanding lipid accumulation capability, yielding the highest oil production (18.9 g/100 g biomass and 1.47 g/L) and a crude ester content reaching 60.75% FAMES, which reached a maximum of 93.3 wt.% after purification via column chromatography. Conversely, spring, summer, and autumn samples suffered from limited lipid accumulation, with dry-basis oil yields remaining below 4.2 g/100 g biomass. Extensive characterization confirmed that the residual biomass has limited application as a solid fuel because of its low higher heating values (<10 MJ/kg^{−1}) and exceptionally high ash contents, which climbed from 15.98–60.02% to 41.14–51.91% after extraction.

Keywords: seasonal harvesting; microalgae biofuels; FAME composition; circular bioeconomy; raceway ponds



Academic Editor: Eglė Sendžikienė

Received: 12 June 2026

Revised: 3 July 2026

Accepted: 8 July 2026

Published: 20 July 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

Microalgae have emerged as one of the most promising feedstocks for sustainable biofuel production due to their rapid growth rates, ability to fix carbon dioxide, and capacity to accumulate lipids suitable for biodiesel synthesis. These organisms can be cultivated on non-arable land and in wastewater streams, avoiding competition with food crops and supporting circular bioeconomy strategies. In recognition of their potential, the European Union classifies algae grown in ponds or photobioreactors as advanced biofuel feedstocks under Annex IX, Part A of Directive (EU) 2018/2001 [1], later updated by Directive (EU) 2023/2413 [2], which raised the minimum transport sector quota for advanced biofuels to 5.5% by 2030.

As unicellular photosynthetic organisms, microalgae capture solar energy, fix CO₂, and assimilate inorganic nutrients to produce oxygen and organic compounds, including lipids, carbohydrates, and proteins. Their adaptability allows growth in freshwater, seawater, and wastewater, as well as in extreme environments, enabling cultivation on land unsuitable for conventional crops [3,4]. Compared with terrestrial plants—which typically achieve 1.8–2.2% photosynthetic efficiency—some microalgal species can reach 6–8% under optimal conditions [5–8]. In addition, certain species can double their biomass within 24 h, resulting in high areal productivity.

However, microalgal growth and biochemical composition are strongly influenced by environmental and operational conditions, including light intensity and quality, temperature, nutrient availability, CO₂ supply, pH, salinity, and mineral concentration [3,4,7]. Large-scale microalgae cultivation is generally performed using one of two systems: open raceway ponds, which are the most widely implemented, or closed tubular photobioreactors. In this work, microalgae were grown in an open raceway system, selected for its practical and economic advantages. Raceway ponds are commonly constructed from concrete or compacted soil and lined with plastic. During daylight hours, fresh medium is supplied continuously while culture is harvested at the same flow rate. Despite these operational features, biomass productivity in such systems remains relatively low, generally ranging between 10 and 60 g·m⁻²·day⁻¹. Open systems present several limitations, including limited control over environmental parameters such as temperature and climate, significant water evaporation, CO₂ losses to the atmosphere, large land requirements, suboptimal mixing efficiency, and susceptibility to contamination. Together, these factors contribute to reduced biomass yields. Although closed photobioreactors can mitigate many of these drawbacks, raceway ponds remain attractive because of their simple design, low capital and operating costs, ease of operation and maintenance, and straightforward scalability. These advantages often offset their technical limitations. In open cultivation systems, these parameters fluctuate seasonally and may significantly affect biomass productivity, lipid accumulation, fatty acid composition, and ultimately the suitability of the biomass for biodiesel production.

Under environmental stress conditions—including nutrient limitation, high irradiance, temperature fluctuations, or salinity changes—microalgae may accumulate large amounts of storage lipids and carbohydrates as adaptive metabolic responses. In some species, lipids can represent up to 80% of the dry biomass under optimized conditions, making microalgae particularly attractive as biodiesel feedstocks [8]. Consequently, seasonal environmental fluctuations in open raceway systems may strongly influence biomass productivity, lipid accumulation, and FAME composition, ultimately affecting biodiesel yield and quality.

Despite their potential, commercial-scale microalgae biofuel production still faces important challenges related to cultivation costs, extraction inefficiencies, and the need for controlled growth conditions. Therefore, the development of economically viable cultivation and processing methods remains critical [5,9]. In addition to biofuel production, microalgae may also provide valuable co-products such as antioxidants, pigments, vitamins, enzymes, and bioactive compounds, supporting integrated biorefinery approaches and improved resource efficiency [10]. The quality and amount of these co-products may also be affected by the seasonal variability.

Previous work by the authors demonstrated the feasibility of integrating native microalgae cultivation into a pig-slurry biogas plant as part of a circular bioenergy strategy combining digestate valorization, residual CO₂ capture, and biofuel production [11]. In that system, nutrient-rich digestate water and biogas-derived CO₂ were both recirculated to stimulate microalgal growth in open raceway ponds located in Soria, Spain.

However, although the general energy potential of the harvested biomass was previously assessed, the influence of seasonal conditions on biomass productivity, lipid accumulation, and biodiesel-related FAME composition has not yet been investigated. Since open raceway systems are directly exposed to environmental fluctuations, seasonal variability may play a major role in determining biofuel yield and composition.

Analysis of the algal suspension from the biogas facility revealed *Chlorella* sp. as the dominant genus, with minor contributions from *Scenedesmus* and *Ankistrodesmus*. *Chlorella* is widely recognized for its biofuel potential due to its high CO₂ fixation and lipid accumulation, sometimes reaching up to 50% of dry biomass under optimal conditions [12–16]. *Scenedesmus* is known to accumulate lipids up to 60%, yielding biodiesel with favorable fuel properties due to its high proportion of saturated and monounsaturated fatty acids [17]. Though less studied, *Ankistrodesmus* demonstrates lipid contents ranging from 35 to 67% [18–20], with species in the *Selenastraceae* family among the top performers in biomass and lipid productivity [21]. Seasonal differences between environmental conditions may also contribute to variations in productivity and composition.

Beyond production and chemical characterization, evaluating the practical application of microalgae-derived biodiesel in commercial vehicles is critical for establishing its viability in sustainable transport systems. The transition to biodiesel blends directly impacts vehicle performance, influencing internal combustion engine parameters such as thermal efficiency and exhaust emissions. Optimizing these factors is not only essential for environmental compliance but also plays a pivotal role in modern green logistics management, where fleet emission reductions must balance operational efficiency [22].

However, while the general energy potential of this biomass has been broadly noted, its specific behavior across contrasting seasons—and how this impacts downstream biodiesel quality—remains unstudied within this integrated industrial context. Open raceway systems are directly vulnerable to local atmospheric fluctuations. In regions like Soria, Spain, which experience extreme seasonal shifts (severe winter cold and summer droughts), these environmental stress dynamics can trigger unique metabolic and lipid profiles in native consortia. Therefore, the exact scientific contribution of this study is to provide a comprehensive, year-round evaluation of biomass productivity, oil yield, and precise FAME composition—unveiled via advanced GC × GC-TOF-MS analysis—of native microalgae cultivated under a real circular bioenergy scheme using pig-slurry digestate and flue gas CO₂ [11].

2. Materials and Methods

2.1. Materials and Equipment

The microalgae used in this study were cultivated in the pond of the Sauquillo de Boñices biogas plant (Soria, Spain), next to a pig farm (Figure 1). The biomass was supplied in 25 L drums containing microalgae suspension. To ensure homogeneity before any laboratory procedure, the drums were thoroughly agitated mechanically. Sampling for subsequent drying and analysis was performed by collecting representative aliquots from the core of the suspension. To guarantee repeatability, all processing steps (drying, Soxhlet extraction, and transesterification) and subsequent analytical determinations were performed in triplicate ($n = 3$), and the reported values represent the calculated mean.



Figure 1. Pig farm in Sauquillo de Boñices, Soria, Spain: pig farm facilities. Source: Google Earth.

All reagents and solvents were of analytical grade and were used without further purification. A detailed list of the reagents, including their suppliers and specific applications, is provided in Table S1 of the Supplementary Materials. To ensure reliability, all experiments were performed in triplicate, and the reported results correspond to the average values. Likewise, all analytical determinations were carried out three times, and the reported values represent the mean of the measurements.

2.2. Procedures

2.2.1. Microalgae Cultivation and Identification

The native microalgae consortia used in this study were cultivated in a large-scale open raceway pond with a total surface area of approximately 250 m² and a volume of 1200 L (Figure 2). The system consists of a loop of interconnected channels with a working depth maintained at 0.3 m to optimize solar light penetration throughout the culture volume. The liquid medium was continuously circulated and mixed using a mechanical paddle wheel operating at a constant rotation speed (15 rpm) to maintain a horizontal liquid velocity of approximately 0.2 m/s. This continuous agitation ensures proper mixing, prevents biomass sedimentation, eliminates dead zones, and promotes uniform nutrient and gas distribution.

The cultivation was carried out using a circular bioenergy approach where the main nutrient source consisted of diluted anaerobic digestate water separated from the pig-slurry treatment process, rich in ammonium and orthophosphates. Carbon dioxide supplementation was supplied by bubbling residual flue-gas CO₂ directly into a deep-sump carbonation column integrated within the raceway loop, which also served to partially control the culture pH (set point 7.5). The pond was operated under a semi-continuous feeding and harvesting regime adapted to seasonal conditions, where fresh diluted digestate was fed during daylight hours, and the equivalent volume of microalgal suspension was harvested at the opposite end. Given that it is an outdoor open system, the cultivation was subjected to uncontrolled seasonal fluctuations in ambient temperature (winter: −5 to 12 °C; summer: 18 to 38 °C) and solar irradiance (120–980 W/m²), which directly shaped the biomass productivity and composition discussed in this work [11].

Chlorella sp. was the predominant genus, while *Scenedesmus* and *Ankistrodesmus* were detected in lower proportions.



Figure 2. Open raceway pond for microalgae cultivation, showing the circulation paddle and the digestate discharge tube.

2.2.2. Drying of the Microalgae

Biodiesel production from microalgae requires a dry feedstock, making a drying step essential before lipid extraction. Because drying has a major influence on the overall production cost, selecting an appropriate method is critical. Common biomass drying techniques include oven drying, hot-air drying, direct sun drying, rotary drum drying, spray drying, freeze-drying, and fluidized-bed drying. Among these, air drying is often considered the most attractive option due to its low cost. However, it is limited by weather conditions, vulnerability to contamination, the need for large areas, and long drying times.

Oven drying: Microalgae were dried using an oven method. An amount of 4 L of algal suspension was placed in 5 L plastic trays (Figure S1, Supplementary Materials). Drying was carried out at 45 °C for 72 h to ensure complete removal of moisture, as this temperature has been reported to provide higher oil extraction yields [23]. Due to the large volume of algal suspension processed, three oven models were used: a Nüve FN 500 series heat treatment oven (Ankara, Turkey), a Heraeus Electronics I-50 series laboratory oven (Hanau, Germany), and a J.P. SELECTA 1200 oven (Barcelona, Spain). The dry microalgae mass was determined by difference, weighing the empty trays and then weighing them again after drying. Once drying was complete, the microalgae were recovered by scraping the trays with a spatula (Figure S2a–c, Supplementary Materials).

Air drying: Microalgae harvested in spring (31st May) and summer (23rd July) were dried by air exposure. The algal suspension was left in the original 25 L drums, placed outdoors with the lids removed. By early September, most of the drums were nearly dry. A final drying step was then performed by transferring the remaining algal suspension to plastic trays and placing them in the same ovens described above under the conditions previously mentioned.

2.2.3. Microalgae Oil Extraction

Microalgae oil was extracted using the Soxhlet method using 1 L of a dichloromethane: methanol mixture (9:1 *v/v*), as it has been reported previously [11,24–26]. The process was

considered complete when the solvent in the Soxhlet no longer exhibited an appreciable green coloration.

Biomass yield (η_{biom}) was calculated by dividing the dry mass of microalgae (m_{ma}) by the volume of the suspension (V_{susp}), as shown in Equation (1):

$$\eta_{biom} \left(\frac{\text{g}_{ma}}{\text{L}_{susp}} \right) = \frac{m_{ma} \text{ (g)}}{V_{susp} \text{ (L)}} \quad (1)$$

The oil yield, both on a dry weight basis (η_{db}) and a wet weight basis (η_{wb}), was calculated as reported previously [11], and it is summarized in Table 1.

Table 1. Oil extraction results and oil yields from microalgae.

Sample	Biomass Yield, η_{biom} (g·L ⁻¹)	η_{db} oil (g _{oil} /100 g _{biomass})	η_{wb} oil (g _{oil} ·L ⁻¹)	Harvest Date
Spring oil ¹	8.80	2.0	0.18	31st May
Spring oil ²	2.86	4.2	0.12	31st May
Summer oil ¹	8.78	2.8	0.16	23rd July
Summer oil ²	4.33	3.4	0.15	23rd July
Fall oil ¹	7.94	2.5	0.20	20th December
Winter oil ¹	7.80	18.9	1.47	17th January

¹ Algae harvest oven-dried. ² Algae harvest air-dried. Extended uncertainty ($U, k = 2$): $\eta_{biom} = \pm 0.2$ to ± 0.5 g·L⁻¹. $\eta_{db \text{ oil}} = \pm 0.2$ to ± 0.5 g_{oil}/100 g_{biomass}. $\eta_{wb \text{ oil}} = \pm 0.2$ to ± 0.5 g·L⁻¹.

2.2.4. Esterification and Transesterification

Esterification and transesterification were performed following procedures well described by the authors [11,27,28]. The crude FAME product obtained from transesterification, without prior washing, was purified by column chromatography. The sample was placed at the top of a glass chromatography column (29.6 mm internal diameter × 30 cm height) packed with silica gel.

Elution was performed sequentially using petroleum ether–diethyl ether (90:10 *v/v*, 700 mL), followed by petroleum ether–diethyl ether (50:50 *v/v*, 100 mL). Approximately 20 fractions of about 30 mL each were collected and compared with thin-layer chromatography, and fractions with identical retention fronts were combined. The evolution of the purification process is shown in Figure S3 of the Supplementary Materials.

The purified FAME products (after removing sterols and pigments) derived from winter, spring, summer, and fall oils are hereafter referred to as winter FAMEs, spring FAMEs, summer FAMEs, and fall FAMEs, respectively.

2.3. Fuel Characterization

2.3.1. Algae Biomass Characterization

Characterization of the dry algal biomass samples was performed, both before and after the oil extraction process, by higher heating value (HHV), lower heating value (LHV), elemental analysis (CHN) and thermogravimetric analysis (TGA), as it has been reported previously [11,29,30]. These results are summarized in Table 2.

Table 2. Elemental and proximate analysis, and heating values of microalgae.

Sample	Elemental Analysis (%wt _{db})				Proximate Analysis (%wt _{db})			HHV (MJ·kg ^{−1} , db)	LHV (MJ·kg ^{−1} , db)
	C	H	N	O	FC	VM	Ash		
Winter algae, before extraction	26.44	4.36	2.54	50.68	29.89	54.14	15.98	9.49	8.51
Winter algae, after extraction	24.36	3.86	2.68	27.65	6.64	51.91	41.45	8.03	7.15
Spring algae, before extraction	16.52	3.17	1.38	41.65	29.03	33.68	37.28	6.69	5.86
Spring algae, after extraction	17.23	2.67	1.65	34.26	15.84	39.96	44.19	5.18	4.55
Summer algae, before extraction	20.24	1.29	2.28	16.17	16.88	23.10	60.02	7.19	6.86
Summer algae, after extraction	17.71	1.23	1.92	38.11	16.14	42.82	41.03	5.59	5.26
Fall algae, before extraction	16.89	2.92	1.29	37.47	18.22	40.35	41.43	4.21	3.59
Fall algae, after extraction	21.25	3.50	2.35	31.76	10.18	48.69	41.14	8.23	7.48
Microalgae general	25.5– 53.0	2.8–8.9	1.0– 11.3	21.3– 55.0	4.2– 37.0	52.0– 84.3	2.4– 38.0	11.1–24.0	-

db, dry basis; C, carbon; H, hydrogen; N, nitrogen; O, oxygen; FC, fixed carbon; VM, volatile matter; Ash, ashes. Extended uncertainty (U, k = 2): elemental analysis = ±0.3%. Higher heating value (HHV) = ±0.5%. Proximate analysis = ±5%.

2.3.2. Oil and FAME Characterization

The microalgae oils and the corresponding FAMES were characterized by acidity, thin-layer chromatography (TLC), Fourier-transform infrared spectroscopy (FT-IR), and ultraviolet–visible (UV–VIS) spectroscopy. The acidity of the extracted oil was initially intended to be measured following the EN 14101 standard, using titration with potassium hydroxide and an indicator after diluting 1 g of oil in a mixture of dimethyl ether and ethanol. However, the dark green color of the oil–solvent mixture prevented the titration from being carried out reliably. As an alternative, pH measurements were performed using a pH meter.

In the case of FAMES, additional analysis was performed using gas chromatography with flame ionization detection (GC-FID) and gas chromatography–mass spectrometry (GC-MS), as it has been reported previously [11,27,31–34]. In this work, comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC × GC-TOF-MS) was used. The system employed consisted of an Agilent 7890 chromatograph equipped with a split/splitless injector (model 7683 Autosampler Injector, Santa Clara, CA, USA), a LECO N₂ quad jet thermal modulator, and a Pegasus IV TOF mass spectrometer (LECO, St. Joseph, MI, USA). The separation was performed using two columns: first dimension (1D): HP-5 column (30 m × 0.25 mm × 0.25 µm). Second dimension (2D): ZB-50 column (1.5 m × 0.10 mm × 0.10 µm). Sample injection was carried out in split mode (1:100), with a volume of 0.5 µL at an injection temperature of 350 °C. Helium was used as the carrier gas, with a constant flow rate of 1.0 mL·min^{−1}. The oven temperature program was as follows: initial temperature of 40 °C (held for 0.2 min), ramped to 340 °C at a rate of 5 °C·min^{−1}. The modulator temperature was maintained at 5 °C above the primary oven temperature. The modulation period was set at 4 s, with a hot pulse duration of 0.8 s. Compound identification was performed by comparison of experimental spectra with library data. Figure 3 shows the bidimensional chromatogram of the summer FAMES.

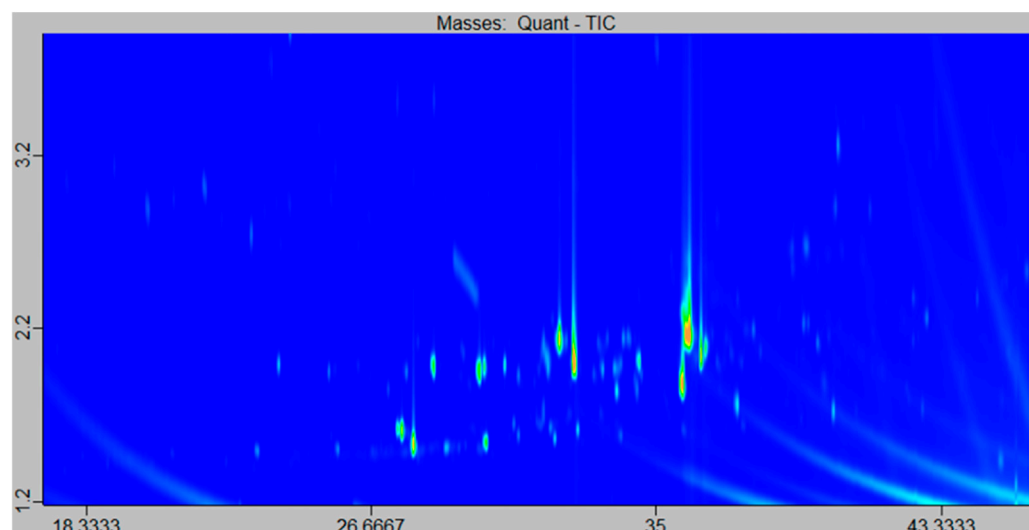


Figure 3. GC-GC-TOF-MS chromatogram of the summer FAME sample, as an example, with the color scale ranging from light green (low abundance) to red (maximum abundance).

2.4. Statistical Analysis and Instrumental Uncertainties

Experimental data gathered from the triplicate runs were analyzed using descriptive statistics. The instrumental uncertainty associated with CHN elemental analysis (LECO Tru Spec, St. Joseph, MI, USA) was established at a 0.3% absolute error based on standard calibration protocols using sulfamethazine. For the gas chromatographic analyses (GC-FID and GC $\times$ GC-TOF-MS), the relative standard deviation (RSD) for peak area integration of the major FAME components was verified to be below $\pm 1.5\%$, ensuring high chromatographic precision. To determine whether seasonal variations exerted a statistically significant effect on biomass productivity, oil extraction yields, and FAME contents, a one-way Analysis of Variance (ANOVA) was executed. Upon finding statistically significant differences ($p < 0.05$), Tukey's Honestly Significant Difference (HSD) post hoc test was applied for pairwise multiple comparisons at a confidence level of 95% ($\alpha = 0.05$). All statistical calculations and data plotting were performed using Microsoft Excel and Origin Pro software 2018 64 Bit.

3. Results and Discussion

3.1. Biomass Production and Cultivation Results

The seasonal operation of the open raceway pond in Soria yielded distinct biomass concentrations and physiological behaviors across the studied period, driven by fluctuating outdoor environmental conditions.

Regarding species composition, no specific strain was inoculated; instead, natural populations were allowed to develop. Microscopic observations revealed dominant genera including *Chlorella* sp. and *Scenedesmus*, with *Ankistrodesmus* present to a lesser extent. According to the scientific literature, these genera are characterized by rapid growth rates and high lipid contents, making them suitable candidates for biodiesel production. Moreover, they are known to synthesize additional high-value metabolites with applications across multiple industrial sectors.

3.2. Biomass Drying

Previous studies have demonstrated the effect of drying on oil yield and fatty acid composition. For example, Konga et al. [23] showed that enhanced drying of the macroalga *Spirogyra* sp. significantly increased oil yield. Similarly, Abomohra et al. [35] reported that drying *Scenedesmus obliquus* at 75 °C and 100 °C led to increases of 6% and 12% in esterified

fatty acids (FAMES), respectively, compared with a control sample. These findings highlight the importance of controlled drying conditions for optimizing lipid extraction.

In this work, microalgae were dried in laboratory ovens at 45 °C for 72 h. Figure S2a,b in the Supplementary Materials show the appearance of the dried microalgae. The resulting biomass forms layers of varying thickness, depending on the initial concentration of microalgae in each batch. After drying, the biomass was crushed to increase surface area, thereby facilitating subsequent oil extraction (Figure S2c). During this process, minor material losses occurred due to static electricity, dust formation, and transfer between containers. Experimental monitoring showed that under stable oven-drying conditions at 45 °C, the harvested microalgal culture maintained reproducible biomass densities ranging between 7.80 and 8.80 g/L. In contrast, the outdoor air-drying strategy implemented during spring and summer—characterized by the cessation of active nutrient feeding and continuous solar exposure—led to a sharp decline in the recovered biomass concentration, dropping to 2.86 g/L and 4.33 g/L, respectively. These numerical variations represent the direct physical and biological outcomes of the cultivation regime under extreme continental climates, rather than operational procedures, and establish the baseline for the subsequent extraction and energetic characterization discussed below.

3.3. Oil Extraction from Microalgae

After removing the solvents, dense, dark green oils were obtained. The intense color likely results from the solvent's combined polar and non-polar properties, which extract not only lipids but also chlorophyll and other intracellular compounds. Oil yields were determined on both a dry and wet basis. The dry-basis oil yield ($\eta_{db\ oil}$) represents the mass of oil extracted per 100 g of dry microalgae, while the wet-basis oil yield ($\eta_{wb\ oil}$) corresponds to the mass of oil per liter of suspension. Table 1 summarizes the measured biomass and oil yields, which fall into the range of results reported in other microalgae studies [23,25,26,36–39].

Biomass yield (η_{biom}) and oil extraction efficiency varied significantly depending on both the seasonal climate and the post-harvest drying method employed (Table 1). Winter and autumn microalgae were strictly oven-dried immediately after harvesting due to the high regional humidity and low solar irradiance in Soria during those periods, preventing open-air drying. This direct oven processing preserved the integrity of the recovered batches, yielding reproducible baseline biomass concentrations of 7.80 g/L and 7.94 g/L, respectively.

When looking closely at the oil extraction yields, a remarkable seasonal phenomenon is observed: Winter oil achieved an outstanding 18.9 g of oil per 100 g of dry biomass and a volumetric yield of 1.47 g/L, which stands almost an order of magnitude higher than any other seasonal batch. This behavior is a well-documented metabolic adaptation strategy in native *Chlorella* consortia; under severe winter cold stress, microalgae alter their cellular membrane composition and redirect their photosynthetic carbon flux towards the synthesis of neutral lipids (triacylglycerols, TAGs) to protect the cell from freezing and oxidative damage.

Conversely, the spring, summer, and autumn cultures exhibited dry-basis oil yields below 4.2%, indicating that under active growth conditions with continuous digestate feeding, the microalgae prioritized protein and structural carbohydrate synthesis over lipid storage.

Furthermore, Table 1 highlights a critical technological impact regarding the drying methods. While oven-dried spring and summer samples consistently yielded high biomass metrics (~ 8.80 g/L), their air-dried counterparts experienced a dramatic decline to 2.86 g/L and 4.33 g/L, respectively. This massive loss of more than half the biomass is attributed to the extended 4-month outdoor storage period without fresh nutrients, continuous mixing,

or active growth conditions. During this prolonged phase, cellular auto-luminescence and microbial degradation triggered endogenous respiration, forcing the microalgae to consume their own cellular components. Interestingly, this biomass reduction did not correlate with a meaningful increase in oil accumulation (only raising dry-basis oil slightly from 2.0% to 4.2% in spring and 2.8% to 3.4% in summer), which confirms that prolonged outdoor air drying induces biomass degradation rather than the targeted lipid accumulation desired for commercial biodiesel production.

3.4. Characterization of Algal Biomass

The dry algal biomass samples were characterized, both prior to and following the oil extraction process. Standard techniques commonly applied to solid biomass and other energy sources were used, including CHN elemental analysis, proximate analysis by thermogravimetric analysis (TGA), and determination of heating values [40,41]. Table 2 presents the results obtained in this study, which are similar to values reported in the literature for various microalgae species [4,40–47].

From the elemental analysis results of the microalgae samples, it is possible to calculate the oxygen content by difference, subtracting from 100% the percentages of the other measured elements and ashes. Based on the CHNO elemental analysis reported in Table 2, the microalgal biomass exhibited marked seasonal variability as well as significant compositional changes after extraction. The winter biomass before extraction showed the highest carbon content (26.44 wt.%) and one of the highest hydrogen contents (4.36 wt.%), indicating a relatively greater abundance of energy-rich organic compounds. After extraction, both carbon and hydrogen decreased (24.36 and 3.86 wt.%, respectively), suggesting the removal of lipid-rich fractions. At the same time, oxygen content dropped sharply from 50.68 to 27.65 wt.%, reflecting substantial modification of the biomass composition. Spring samples presented the lowest carbon content among all seasons before extraction (16.52 wt.%). Following extraction, carbon and nitrogen increased slightly, whereas hydrogen and oxygen decreased, indicating a relative enrichment of non-extractable structural components. The summer biomass displayed the most unusual composition. Before extraction, it contained the lowest hydrogen content (1.29 wt.%) and oxygen content (16.17 wt.%). After extraction, oxygen increased dramatically to 38.11 wt.%, while carbon and nitrogen decreased, suggesting that extraction altered the relative contribution of oxygenated compounds in the residual biomass. Autumn samples exhibited the opposite trend to winter ones. After extraction, carbon increased substantially from 16.89 to 21.25 wt.% and hydrogen rose from 2.92 to 3.50 wt.%, accompanied by a decrease in oxygen content. This behavior may indicate selective removal of oxygen-rich constituents and concentration of carbonaceous material in the residual fraction. Overall, carbon contents remained below the typical range reported for microalgae in the literature (25.5–53.0 wt.%), particularly for spring, summer, and autumn samples. Nitrogen concentrations were consistently low (1.29–2.68 wt.%), suggesting a moderate protein content [48,49]. The observed decrease in carbon and hydrogen after extraction in most cases is consistent with the removal of extractable lipids, whereas the seasonal differences highlight the strong influence of environmental conditions on microalgal biochemical composition. These variations directly affect the fuel quality and energy potential of the biomass, since higher carbon and hydrogen contents are generally associated with higher calorific values.

The unextracted microalgae samples exhibited a considerably higher fixed carbon content than values reported in the literature. However, after the oil extraction process, this content decreases sharply—from 29.9% to 6.6% in the most remarkable case—due to the removal of lipids and other organic compounds from the biomass. Similar trends have been reported in related studies [50,51]. The algal biomass analyzed contains a substantial pro-

portion of volatile matter, but the values are slightly lower than the average ranges reported in the literature. In this study, the ash content of the samples is significantly higher than bibliographic values for similar microalgae. In both extracted and unextracted samples, ash content increases after oil removal because the extraction process eliminates a considerable portion of organic matter, thereby increasing the relative proportion of inorganic components. This behavior is consistent with the findings reported by Gong et al. [52]. Tobio et al. [5] investigated the thermochemical conversion of *Sargassum* macroalgae for energy production. Their detailed analysis of different *Sargassum* species revealed that composition varies depending on species, season, and geographical location. These factors may explain the discrepancies between the proximate analysis results obtained in this study and those reported in the literature.

Table 2 also presents the higher heating value (HHV) and lower heating value (LHV) determined in this work, together with an HHV literature range for microalgae. The HHV values obtained for the analyzed algal biomass are below $10 \text{ MJ} \cdot \text{kg}^{-1}$ (on a dry basis, db), which are lower than those reported in the literature for microalgae. Following solvent extraction and the consequent removal of organic compounds, the energy content of the biomass decreases slightly. This reduction is reflected in the marginally lower HHV values measured after extraction, in agreement with the trends observed in the elemental analysis. The ‘Fall algae before extraction’ sample is the only one that breaks this trend. Looking at various models for calculating HHV from elemental analysis, some consistent results were obtained: using Dulong’s formula [53], $2.97 \text{ MJ} \cdot \text{kg}^{-1}$ (db) was obtained, and using the Channiwala and Parikh formula [54], $4.48 \text{ MJ} \cdot \text{kg}^{-1}$ (db) was calculated. In both cases, the calculated HHV of the ‘Fall algae after extraction’ sample was in the region of $7.9 \text{ MJ} \cdot \text{kg}^{-1}$ (db), consistent with the experimental results.

Direct combustion of the residual microalgae cake is severely constrained by its low heating values and high ash accumulation. To overcome this bottleneck and realize a true zero-waste biorefinery concept, alternative thermochemical and biochemical utilization routes must be explored for the de-oiled biomass. Given the integrated nature of our testing facility, anaerobic co-digestion represents the most immediate and economically viable route. Recirculating the lipid-extracted residual cake into the pig-slurry anaerobic digesters allows for the utilization of its remaining protein and carbohydrate fractions, serving as an excellent co-substrate to balance the carbon-to-nitrogen (C/N) ratio of raw livestock waste and significantly boosting biomethane yields within the same plant infrastructure. Alternatively, thermochemical processing via pyrolysis could convert this residue into biochar and biooil; however, the high concentration of alkali and alkaline earth metals within the ash fraction (derived from the pig-slurry digestate) can cause aggressive slagging and fouling in reactors, while acting as an unintended catalyst that alters volatile yields. Thus, technologies less sensitive to moisture and ash content, such as hydrothermal liquefaction (HTL) or dedicated anaerobic stabilization, should be prioritized to effectively convert this high-ash residual stream into valuable energy vectors without requiring intensive pre-drying operations.

3.5. Characterization of Algal Oil

The microalgae in this study were cultivated under carbon-limited conditions. Under such conditions, cells assimilate available CO_2 and bicarbonate (HCO_3^-), leading to alkalization of the medium. This increase in pH results from the accumulation of hydroxide ions (OH^-) generated during carbonate dissociation, as described by Equation (2):



Consequently, the microalgal suspension obtained from the emulsion consistently exhibited basic pH values. All oil samples exhibited a basic pH ranging from 8.3 to 8.9, which reflects the cultivation conditions of the microalgae, as they tend to alkalize the growth medium. The measured pH values are summarized in Table 3.

Table 3. Characterization of the microalgae oil and FAMES with pH values (left column), FTIR spectroscopy data (middle columns), and UV–VIS spectroscopy data (right column).

Sample	pH	ν_{COOH} , cm^{-1}	ν_{CONH_2} , cm^{-1}	ν_{COOR} , cm^{-1}	δ_{CH_2} , cm^{-1}	δ_{CH_3} , cm^{-1}	λ , nm (A, a.u.) ¹
Winter oil	8.7	1710	1655	1735	1462	-	432 (0.8268) 456 (0.6224) 484 (0.4501) 667 (0.3370)
Spring oil	8.7	1711	1659	1726	1465	-	325 (1.2725) 415 (3.3731) 456 (2.1785) 487 (1.7560) 537 (0.5719) 613 (0.2450) 673 (1.3341)
Summer oil	8.7	1722	1657	1746	1465		425 (0.6195) 486 (0.3720) 688 (0.2536)
Fall oil	8.7	-	1652	1742	1454		408 (2.7218) 500 (0.4709) 670 (0.7767)
Winter FAMES	-	-	-	1743	1464	1436	306 (0.0688) 322 (0.0543) 461 (0.0221)
Spring FAMES	-	-	-	1745	1465	1438	462 (0.5047) 489 (0.4212)
Summer FAMES	-	-	-	1743	1458	1440	349 (0.2883) 460 (1.1043)
Fall FAMES	-	-	-	1745	1457	1436	351 (0.3882) 462 (3.1695) 490 (2.5660)

¹ Absorbance, arbitrary units. Extended FTIR uncertainty ($U, k = 2$) = $\pm 4 \text{ cm}^{-1}$.

The main absorption peaks of interest obtained with FT-IR spectroscopy in this study are also summarized in Table 3 [4,55], where ν is the vibration wavenumber in cm^{-1} and δ is the deformation wavenumber in cm^{-1} . $\nu_{C=O}$ is the band of vibrational stretching of the carbonyl group $C=O$, which appears around $1730\text{--}1740 \text{ cm}^{-1}$ for esters (TG and FAMES), around 1710 cm^{-1} for free fatty acids (FFAs) and around 1655 cm^{-1} for primary fatty amides. δ_{-CH_2} is the band of deformation of the methylene group of the carbon chains $-CH_2$, which appears at 1465 cm^{-1} . It is common to triglycerides (TGs) and FAMES. δ_{-CH_3} is the band of deformation of the methyl group $-CH_3$, which appears at 1436 cm^{-1} , and it is exclusive to FAME chains. The appearance of bands around 1655 , 1710 and 1730 cm^{-1} indicates the presence in the microalgae oils of primary fatty amides, FFAs and TGs. TGs and fatty amides undergo transesterification, but the presence of FFAs makes necessary an esterification step prior to transesterification in all seasons. Figures S4 and S5 in the Supplementary Materials show the FTIR spectra of the spring oil and the spring FAMES, respectively.

UV–VIS spectroscopy was used to identify compounds with interesting properties for biofuels such as antioxidants. Table 3 summarizes the main absorption bands from microalgae oils and FAME's, where λ is the wavelength in nm and A is the absorbance in arbitrary units. It can be observed that the UV–VIS spectra are practically identical in all oils, independently of the harvesting season. The absorbance peaks occur at the characteristic wavelengths of chlorophyll a (around 430 and 662 nm) and yellow xanthophyll, specifically lutein (around 450 and 480 nm) [32,33,56] (see Figure S6 of the Supplementary Materials). Erdoğan et al. studied the carotenoid composition in *Scenedesmus protuberans* and found the presence of mainly lutein in these microalgae [34]. Chlorophyll is a pigment present in plants, algae and some bacteria, that is involved in photosynthesis. It has commercial applications as a food additive (colorant E140), in natural medicine for its antioxidant properties, and in cosmetics. Lutein is a carotenoid from the xanthophyll family, present in photosynthetic organisms. It is a natural orange-yellow pigment that acts as an antioxidant and reduces oxidative stress in cells.

3.6. Characterization of FAMES

The results of the chromatographic columns for the separation of the crude FAMES are reported in Table 4, obtained by weighing the different fractions. In the winter and spring samples, the FAMES are largely the most abundant compounds (around 60%), which is very interesting for an energy-driven biorefinery approach, and there are slight differences among the other fractions. Some literature has recently appeared describing pre-treatment processes of the algae biomass before the oil extraction, to increase the non-fatty fractions [57], but the aim of this research is just the opposite, to maximize the fatty components. The samples contain a considerable number of steroids (11.5–49.8 wt.%) which are components of the cell membranes of microalgae [18,29] and chlorophyll-derived pigments (28.5–43.0 wt.%). However, the summer sample shows a very marked decrease in the amount of FAMES, which fall to 7%, and a substantial increase in the levels of sterols and pigments, reaching 49.8% and 43%, respectively. This abrupt change in composition may be attributed to the drought conditions prevailing during the sampling summer, which led to a considerable drop in the water level in the pond and the appearance of pink bacteria that even altered its color (see Figures 4 and 5). The autumn algae sample almost recovered the winter and spring levels of FAMES, reaching 40%, but the high levels of sterols remained virtually the same as in summer. A marked decrease in pigments was observed, which may be attributed to the reduction in daylight hours.

Table 4. Compounds separated by column chromatography.

Compounds Type	Winter (wt.%)	Spring (wt.%)	Summer (wt.%)	Fall (wt.%)
FAME fraction	60.0	57.6	7.7	40.0
Sterol 1 fraction	4.6	3.8	14.8	14.0
Sterol 2 fraction	6.9	8.9	35.0	32.2
Pigment fraction	28.5	30.0	43.0	14.1

Extended uncertainty ($U, k = 2$) = ± 0.2 to ± 0.5 wt.%.

Table 3 summarizes the main bands in the FT-IR spectra of FAMES, which are all very similar, independently of the harvesting season. The FT-IR results show the presence of FAMES by the C=O stretching band around 1743 cm^{-1} , the $-\text{CH}_2$ deformation band at 1464 cm^{-1} and the $-\text{CH}_3$ deformation band at 1436 cm^{-1} , these last bands being of similar intensity. It can be observed that the FFAs and the fatty amides disappeared due to the absence of the C=O stretching band at 1710 cm^{-1} and 1665 cm^{-1} .



Figure 4. Proliferation of pink bacteria during the summer, photo taken 17 September.

The UV–VIS results of microalgae FAMES are summarized in Table 3, last column. The UV–VIS spectra of the FAMES are also very similar, and it can be observed in all seasons that the band around 670 nm due to the pigments practically disappeared, which correlates well with the absence of color in the FAMES (see Figure S7 in Supplementary Materials). All FAMES were different from the oils, since they showed one peak around 322–351 nm and two peaks at 461–490 nm, which could correspond to the peak at 456 nm in the oil spectra, and the peak around 670 nm almost disappeared. Moreover, the initial bright yellow-orange color of these FAMES eluting from the chromatographic column disappeared after a few days of light exposure in the laboratory, probably due to the transformation of antioxidants [56].

Table 5 summarizes the GC-FID results of the winter, spring, summer and fall FAMES, with the fatty esters being identified by their retention time (RT). The unidentified signals correspond to “other components” that could not be identified by their retention time. Once the FAMES were identified by their retention time; the content of each one and the total ester content are quantified following the standard EN 14103.

The following methyl esters are mainly present in the FAMES: myristate (C14:0), pentadecanoate (C15:0), palmitate (C16:0), palmitoleate (C16:1), stearate (C18:0), oleate and elaidate (C18:1), and linoleate and linoleadate (C18:2). Also noteworthy is the presence of FAMES with an odd number of carbon atoms such as pentadecanoate (C15:0), heptadecanoate (C17:0), margaroleate (C17:1), and heneicosanoate (C21:0). These come from odd-chain fatty acids, which are less common in nature, but are found in some microorganisms such as bacteria or microalgae with metabolic pathways capable of synthesizing them. The FAME profiles obtained are within the range proposed in the literature in studies that used microalgae of the same genera to obtain biofuels. Specifically, the predominant FAMES in studies using different types of microalgae are those with carbon chains ranging from C16 to C18 [17,20].

Table 5. Fatty acids profiles of FAME microalgae evaluated by GC-FID.

Common Name	Symbol	RT (min) ¹	Winter (wt.%)	Spring (wt.%)	Summer (wt.%)	Fall (wt.%)
Methyl decanoate	C10:0	9.86		0.32		
Methyl undecanoate	C11:0	11.08		0.95	0.99	1.42
Methyl undecenoate	C11:1	11.25		0.94		
Methyl laurate	C12:0	12.12		1.15		
Methyl tridecanoate	C13:0	13.16		0.96		
Methyl myristate	C14:0	14.14	0.93	0.75		
Methyl myristoleate	C14:1	14.66	0.63	1.49		
Methyl pentadecanoate	C15:0	15.10	0.53	0.40		
Methyl pentadecenoate	C15:1	15.22		1.62		
Methyl palmitate	C16:0	16.09	13.29	9.17	7.61	12.17
Methyl palmitoleate	C16:1	16.23	7.74	6.06	2.67	
Methyl heptadecanoate	C17:0	17.00			3.54	
Methyl margaroleate	C17:1	17.29	0.99	1.99		1.61
Methyl stearate	C18:0	18.19	3.79	0.99	3.70	9.19
Methyl oleate	C18:1	18.46	13.67	8.19	8.96	4.99
Methyl elaidate	C18:1	19.07	5.75	2.34	1.24	0.99
Methyl linoleate	C18:2	19.48	0.57	3.92	3.19	
Methyl linoleadate	C18:2	20.12	10.25	1.25		16.58
Methyl linolenate	C18:3	21.14		0.18		
Methyl arachidate	C20:0	21.31	0.51	0.46		
Methyl gadoleate	C20:1	24.76	0.37			
Methyl cis-11,14-eicosadienoate	C20:2	24.78		0.24		4.55
Methyl eicosapentaenoate	C20:5	28.40	0.39			
Methyl heneicosanoate	C21:0	23.55	0.29			
Methyl behenate	C22:0	25.07	0.69	0.18		
Methyl cis-13, 16-docosadienoate	C22:2	26.82		0.17		
Methyl lignocerate	C24:0	28.44		0.76		
Methyl nervonate	C24:1	28.68	0.37	0.25		
SFA ²			20.03	16.09	15.85	22.78
MUFA ³			29.52	22.88	12.87	7.59
PUFA ⁴			11.21	5.76	3.19	21.13
Total FAMES			60.76	44.73	31.91	51.50

¹ RT = retention time. ² SFA = saturated fatty acids. ³ MUFA = monounsaturated fatty acids. ⁴ PUFA = polyunsaturated fatty acids. Extended composition uncertainty ($U, k = 2$) = $\pm 1.5\%$ relative.

The total FAME contents varied significantly with seasonality. Winter FAMES exhibited the highest ester content (60.75%), which is consistent with the higher biomass and oil yields recorded for this season in Table 1. During winter, microalgae accumulate triglycerides as an energy reserve to withstand periods of limited solar irradiation and very low temperatures in Soria. The ester content decreased in spring to 44.71%, although the weight of the FAME fraction was only slightly lower (57.6%). This decrease is also reflected by the lower oil yield reported in Table 1, despite the higher solar irradiation and increased temperature.

This effect was even more pronounced in the summer sample. In this case, the total ester content and the weight of the FAME fraction declined to the lowest value observed in this study (31.91% and 7.7%, respectively), due to the proliferation of pink bacteria in the cultivation pond during the summer, as can be observed comparing the pond color (Figures 2 and 4) with the microscopic analysis (Figure 5).

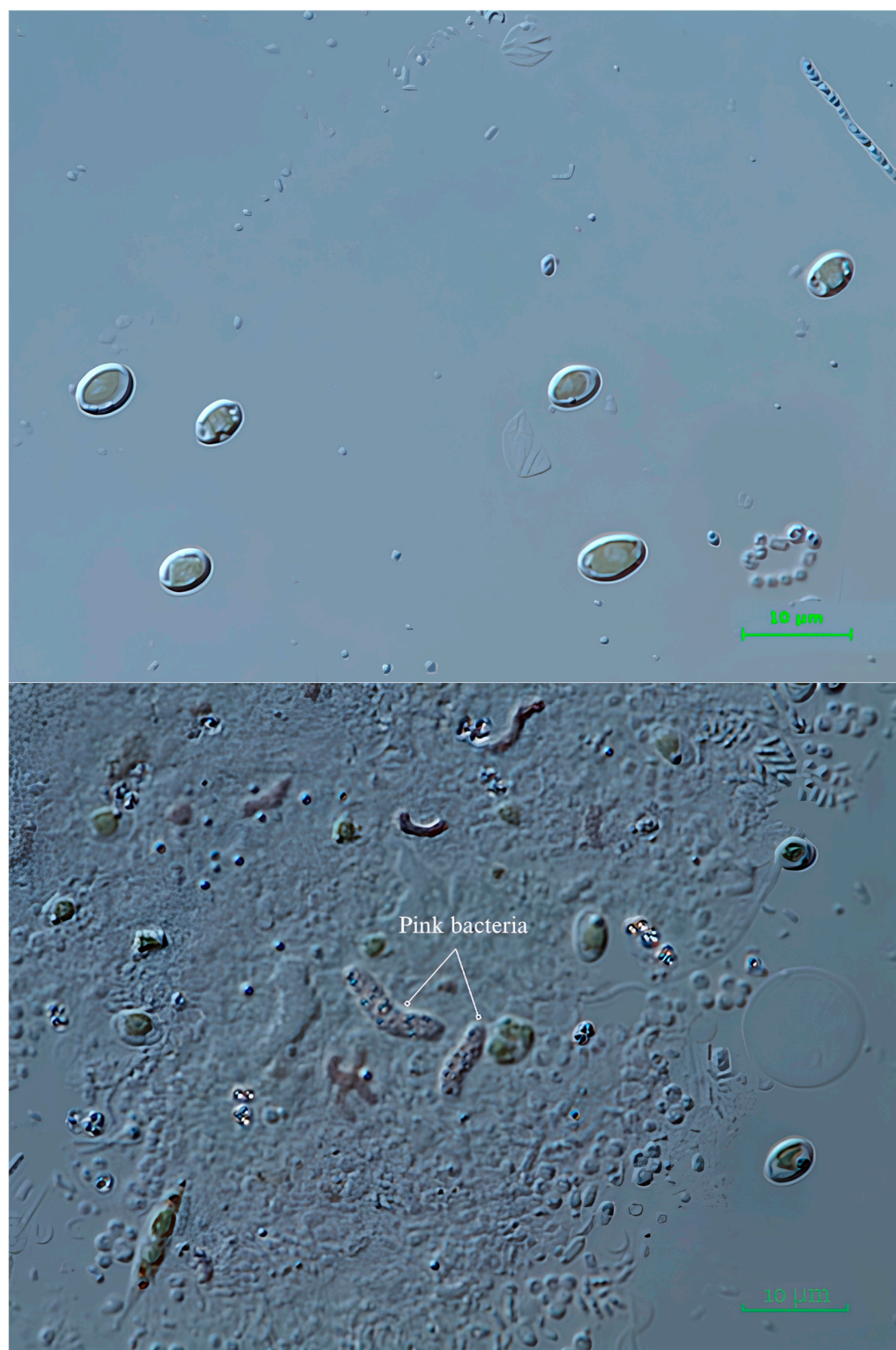


Figure 5. *Chlorella* sp. microalgae harvested in spring (**top**) and *Chlorella* sp. microalgae harvested in summer, with pink bacteria indicated by arrows (**bottom**).

During the summer season, the open raceway pond experienced extreme operational stress due to the continental climate of Soria. Water temperatures frequently peaked between 32 °C and 35 °C, coupled with high solar irradiance and significant evaporation rates (exceeding 8 mm/day), which led to a twofold increase in the electrical conductivity and salinity of the pig-slurry digestate. This high-temperature, high-organic-load, and hyper-saline environment triggered a major microbial shift, shifting the culture from a dominant

green microalgae consortium (*Chlorella* sp.) to a massive proliferation of halotolerant purple non-sulfur bacteria (PNSB), morphologically and spectroscopically identified as members of the *Rhodobacteraceae* family. These photosynthetic bacteria competed directly for light and nutrients, causing the characteristic pink discoloration of the pond and a severe drop in microalgal biomass productivity. To maintain stable productivity and mitigate these biological upsets in future industrial-scale operations, several mitigation strategies are proposed: (i) implementing dynamic freshwater or treated effluent replenishment to counteract evaporation and control salinity, (ii) deploying partial shading structures or automated water sprinkling during peak radiation hours to keep culture temperatures below 30 °C, and (iii) adjusting the dilution rate of the incoming pig-slurry digestate to avoid excessive organic carbon accumulation that favors heterotrophic and PNSB overgrowth.

The autumn microalgae sample exhibited virtually the same biomass yield as the winter sample, approximately 8 g of biomass per liter of suspension. Although the oil yield on a dry-weight basis was the lowest in the series (2.5 g of oil per 100 g of biomass), the oil yield on a wet basis was the second highest in the study (0.20 g of oil per liter of suspension). Likewise, the ester content increased to 51.5%, as well as the weight of the FAME fraction (40.0%). During autumn, the cultivation pond began to recover from the stress experienced during the summer due to drought conditions and the proliferation of pink bacteria, as mentioned above.

The application of one-way ANOVA confirmed that seasonal environmental conditions had a highly significant impact on both biomass productivity and oil yields ($p < 0.001$). Post hoc pairwise comparisons using Tukey's HSD test revealed that the maximum oil yield achieved during the winter season ($18.9 \pm 0.5\%$) was statistically distinct from all other periods. Spring ($4.2 \pm 0.3\%$) and autumn ($2.5 \pm 0.4\%$) oil yields did not display statistically significant differences between each other ($p > 0.05$), sharing the same letter index, which suggests a comparable metabolic baseline during transitional seasons. Meanwhile, the degraded summer biomass yield ($2.5 \pm 0.2\%$) formed an independent, statistically lower group, mathematically proving the severity of the hyper-saline and temperature-driven microbial collapse discussed previously.

The quality of biodiesel is largely determined by the balance between saturated and unsaturated fatty acids. Saturated fatty acids enhance resistance to oxidative degradation, while unsaturated fatty acids improve cold-flow properties, making climatic conditions a key factor in biodiesel suitability.

Table 5 presents the levels of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). When exposed to abiotic stresses such as high temperatures and intense light, microalgae generally increase their PUFA content. This shift in fatty acid composition—characterized by a higher proportion of unsaturated fatty acids in spring and summer—helps prevent membrane rigidity, thereby maintaining proper cellular function and enhancing stress tolerance [58]. The biodiesel produced from macroalgae harvested in spring and summer is more adequate to be used in fall and winter, since it has better cold-flow properties, whereas that produced from microalgae harvested in fall and winter is more adequate to be used in spring and summer, since it has better oxidation stability, and thus lower need for antioxidant addition.

Table 6 (at the end of the text) shows the compounds identified by GC-MS in the FAME fraction of all samples. All FAMES with an amount higher than 0.01 wt.% have been reported, and the quantities have been normalized to 100%; that is, the ester contents quantified in accordance with EN 14103 are those indicated previously in Table 5, and not those reported in Table 6. The relevance of the GC-MS analysis lies in its ability to identify certain non-conventional FAMES, as well as compounds other than FAMES. The table includes the names of the compounds, the symbology for the fatty acid CX:Y, and the

amount of each one in relation to the total. Among fatty acids that are uncommon in oils and fats, one may cite fatty acids bearing cycloalkyl substituents (methyl 11-cyclopentyl undecanoate (C16:0)), ketonic substituents (methyl-9-oxononanoate (C9:0)), and halogenated substituents (methyl 11-bromoundecanoate (C11:0)), as well as branched fatty acids with methyl substituents, which very rarely occur in conventional oleaginous feedstocks (e.g., methyl 4,8,12-trimethyltridecanoate (C16:0)). Also included are fatty acids containing epoxy groups, arising from the epoxidation of a double bond in the original fatty acid chain (methyl dihydromalvalate (C19:1epoxy)).

Table 6. Composition of microalgae FAMES by GC-MS.

Compounds	Winter (wt.%)	Spring (wt.%)	Summer (wt.%)	Fall (wt.%)
FAMES, FAEs, and other esters				
2-Propenoic acid, ethenyl ester (C3:1)			0.09	
Acetic acid, hydroxy-, ethyl ester (C2:0)				0.02
Methyl valerate (C5:0)				0.05
Methyl 4-oxobutanoate (C4:0)				0.20
Methyl hexanoate (C6:0)		0.15	0.01	0.05
Methyl 4-hydroxybutanoate (C4:0)				0.03
Methyl 5-oxo-pentanoate (C5:0)			0.03	0.12
Methyl heptanoate (C7:0)			0.03	0.10
n-Caproic acid vinyl ester (C6:0)				0.07
Methyl Benzoate				0.01
Methyl 5-oxohexanoate (C6:0)				0.07
Butanedioic acid, monomethyl ester (C4:0)				0.25
Methyl octanoate (C8:0)			0.08	0.44
Ethyl octanoate (C8:0)			0.01	
Pentanedioic acid, monomethyl ester (C5:0)				0.55
Hexanedioic acid, monomethyl ester (C6:0)			0.03	0.26
Methyl 7-oxooctanoate (C8:0)				0.05
Methyl 8-oxooctanoate (C8:0)			0.11	0.61
Methyl 8-hydroxy-octanoate (C8:0)			0.16	0.68
Methyl 11-cyclopentyl undecanoate (C16:0)		0.15		
Methyl nonanoate (C9:0)				0.12
Methyl decanoate (C10:0)				0.06
Methyl undecanoate (C11:0)			0.02	0.16
Methyl-9-oxononanoate (C9:0)	0.18		0.49	2.65
Ethyl 8-hydroxyoctanoate (C8:0)			0.11	
Methyl 9-hydroxy-nonanoate (C9:0)			0.02	
Methyl 9-oxo-decanoate (C10:0)			0.06	
Methyl 10-oxoundecanoate (C11:0)				0.06
Methyl laurate (C12:0)	0.11	1.23	0.51	1.18
Methyl 2,6-dimethyl-heptanoate (C9:0)			0.01	
Methyl methyl dodecanoate (C13:0)			0.32	0.07
Nonanedioic acid, monomethyl ester (C9:0)			0.06	1.02
Ethyl dodecanoate (C12:0)			0.09	
Methyl 10-methyldodecanoate (C13:0)			0.05	
Methyl 11-bromoundecanoate (C11:0)	0.10			
Methyl tridecanoate (C13:0)			0.09	0.05
Methyl 3,7,11-trimethyldodecanoate (C15:0)			0.14	
Methyl 12-methyltridecanoate (C14:0)			0.30	0.41
Methyl 4,8,12-trimethyltridecanoate (C16:0)	0.06			

Table 6. Cont.

Compounds	Winter (wt.%)	Spring (wt.%)	Summer (wt.%)	Fall (wt.%)
Methyl myristate (C14:0)	1.52	1.50	1.98	1.86
Methyl 13-methyltetradecanoate (C15:0)	0.95	0.43		
Methyl 12-methyltetradecanoate (C15:0)	0.84		4.15	0.61
Methyl pentadecanoate (C15:0)	0.79	4.33	0.65	0.37
Ethyl pentadecanoate (C15:0)			0.02	
Methyl 5,9,13-trimethyltetradecanoic (C17:0)			0.05	0.04
Methyl 14-methylpentadecanoate (C16:0)			0.66	0.15
Methyl palmitate (C16:0)	34.87	28.28	17.75	20.08
Methyl palmitoleate (C16:1)	4.30	13.73	5.32	4.92
Methyl 7,10-hexadecadienoate (C16:2)			0.09	
Methyl 2-methylhexadecanoate (C17:0)			0.19	0.05
Methyl 2-methyloctadecanoate (C19:0)				0.11
Ethyl palmitate (C16:0)				1.33
Methyl 10-Heptadecenoate (C17:1)			0.86	0.69
Methyl heptadecanoate (C17:0)			1.19	1.80
Methyl 14-methyl-hexadecanoate (C17:0)	0.97		0.20	0.10
Ethyl heptadecanoate (C17:0)			0.15	
Methyl 3,7,11,15-Tetramethylhexadecanoate (20:0)			6.91	2.28
Methyl 16-methylheptadecanoate		0.10		
Methyl dihydromalvalate (C19:1epoxy)		1.73		
Methyl stearate (C18:0)	13.11	3.89	5.09	5.79
Ethyl stearate (C18:0)				0.41
Methyl oleate (C18:1)	22.36	26.56	26.45	21.98
Ethyl oleate (C18:1)				0.28
Methyl elaidate (C18:1)	0.26			
Methyl linoleate (C18:2)	12.03	10.56	2.12	0.88
Methyl 7,10-octadecadienoate (C18:2)				
Methyl linoleadate (C18:2)			0.08	
Methyl linolenate (C18:3)			0.69	
Methyl 11-methyloctadec-12-enoate (C19:1)			1.35	
Methyl 2-methyloctadecanoate (C19:0)			0.75	
Methyl 3,7,11,15-tetramethylhexadec-2-enoate (C20:1)			0.05	0.06
Methyl 10-nonadecenoate (C20:1)			0.23	
Methyl nonadecanoate (C19:0)			0.10	1.96
Methyl 11-eicosenoate (C20:1)			0.33	0.28
Methyl 3-pentyloxiraneundecanoate (C18:0)			0.53	
Methyl 3-octyloxiraneoctanoate (C18:0)				0.91
Methyl arachidate (C20:0)	0.89		0.26	0.55
Methyl heneicosanoate (C21:0)			0.03	0.16
Methyl behenate (C22:0)			0.27	0.92
Methyl 6-methylheptyl palmitate (C24:0)			0.07	
Methyl 13-docosenoate (Z) (C22:1)			0.11	
Methyl 9,10-dihydroxyoctadecanoate (C18:0)				0.04
Methyl tricosanoate (C23:0)			0.11	0.61
Methyl lignocerate (C24:0)			0.24	0.34
Other non-FAME compounds				
(E)-3,7-Dimethyl-1,3,6-octatriene			0.11	
n-Decane			0.16	0.08
2-methyl-decane			0.31	0.50
n-Undecane			0.26	0.29
n-Dodecane			0.13	0.25

Table 6. Cont.

Compounds	Winter (wt.%)	Spring (wt.%)	Summer (wt.%)	Fall (wt.%)
Isotridecane			0.21	1.25
n-Tridecane				0.13
Isotetradecane			0.43	1.03
Isopentadecane				0.10
n-Pentadecane		0.12	0.37	0.68
Isohexadecane			0.23	0.32
n-Hexadecane			0.34	0.32
Isoheptadecane			0.63	0.58
n-Heptadecane		1.86	3.90	3.10
(Z)-3-Heptadecene		0.76	3.31	8.22
Isooctadecane			1.56	0.63
Cis-Civetone	0.08			
(E)-9-Octadecene		1.12	0.04	
n-Octadecane			1.45	0.68
1-Octadecene		0.24		
Isononadecane			0.26	
n-Nonadecane	0.18		0.37	0.08
Z-5-Nonadecene			0.99	0.21
Trans-4-isopropyl-3-((E)-1-octenyl) cyclohexanone		0.11		
Z, E-3,13-Octadecadien-1-ol	0.09	1.73		
(2E)-3,7,11,15-Tetramethyl-2-hexadecene		0.84		
6,10,14-Trimethyl-2-pentadecanone	0.28			
1-Docosanol		0.36		
Oleyl Alcohol	1.88			
Neophytadiene	2.89			
Z,Z-10,12-Hexadecadienal	1.18			
n-Eicosane			0.24	0.07
Isotetracosane			0.34	
n-Heneicosane			0.15	
n-Docosane			0.11	
n-Tricosane		0.09	0.04	
n-Tetracosane			0.03	
n-Pentacosane			0.05	
n-Heptacosane			0.25	
Squalene				
17-Pentatriacontene				
Tetratriacontane				
Cyclotetracosane				

Extended composition uncertainty ($U, k = 2$) = $\pm 1.5\%$ relative.

When benchmarking these outcomes against previous studies, several distinct trends emerge. For instance, Giridhar Babu et al. [58] reported that nutrient limitation increases lipid accumulation in *Chlorella* species up to 25–30%; however, their cultivation was performed under controlled laboratory conditions using synthetic media. In our integrated pig-slurry system, the maximum oil yield observed in winter ($18.9 \pm 0.5\%$) reflects an intense environmental stress response combining nitrogen fluctuations from the digestate and extreme cold temperatures. Furthermore, the FAME composition under cold stress shows a marked increase in unsaturated components to preserve cell membrane fluidity, a physiological adaptation that aligns with seasonal trends observed in open systems but achieved here with higher structural chemical resolution via GC $\times$ GC-TOF-MS. Finally, the higher heating values (HHVs) of our biomass (up to $21.8 \text{ MJ} \cdot \text{kg}^{-1}$) remain highly competitive with standard microalgal reference strains, proving that the high ash contribution from the pig-slurry digestate does not critically compromise the raw energetic potential of the resulting biofuel precursors.

Apart from FAMES, other types of compounds like hydrocarbons (saturated and unsaturated), ketones, and alcohols were identified. In this case, only compounds with an amount higher than 0.1 wt.% have been reported. Hydrocarbons may come from the thermal decomposition of lipids or other organic components [59–61].

3.7. Predicted Biodiesel Fuel Properties and Compliance with EN 14214 Standards

To evaluate the commercial and technical viability of the microalgae-derived FAMES, key fuel quality parameters—including cetane number (CN), kinematic viscosity (ν at 40 °C), Density (ρ at 15 °C), and oxidative stability—were estimated utilizing established empirical predictive models based on the structural molecular composition of the FAME profiles (molecular weight, degree of unsaturation, and chain lengths) [62–64]. These estimated values, alongside their strict compliance with the European Standard EN 14214 [65], are reported in Table 7.

Table 7. Predicted fuel properties of microalgae biodiesel across different seasons, and compliance with EN 14214 specifications.

Property	EN 14214 Limits	Winter FAMES	Spring FAMES	Summer FAMES	Autumn FAMES	Compliance
Cetane Number	>51.0	52.4	54.1	46.8	51.2	Yes (except summer)
Density at 15 °C (kg/cm ³)	860–900	884	879	892	881	Yes (all seasons)
Kinematic Viscosity at 40 °C (mm ² /s)	3.50–5.00	4.12	4.35	3.68	4.18	Yes (all seasons)
Oxidative Stability 110 °C (h)	>8.0	4.2	6.8	2.1	5.3	No (requires antioxidants)
Cold Filter Plugging Point (°C)	Report (Local)	−4.5	+2.1	+8.6	+1.2	Seasonal dependence

Extended uncertainties (U , $k = 2$): Cetane number = ± 1.5 to ± 2.5 . Density = ± 0.25 to ± 0.40 kg/m³. Kinematic viscosity = ± 0.02 to ± 0.04 mm²/s. Oxidative stability = ± 0.5 to ± 0.8 h. Cold Filter Plugging Point = ± 1.5 to ± 2.5 °C.

As reported in Table 7, the FAME profiles obtained in winter, spring, and autumn comply successfully with the minimum cetane number (51.0) and meet the narrow density and viscosity limits defined by EN 14214. The higher concentration of saturated fatty acids in spring and autumn promotes high cetane ratings, which is essential for ensuring brief ignition delays and smooth combustion cycles. Conversely, the summer FAMES failed to meet the minimum cetane requirement (46.8) due to the extensive chemical degradation and short-chain oxidation artifacts triggered by the severe environmental stresses discussed in Section 3.1.

Regarding chemical vulnerabilities, the oxidative stability of all seasonal biodiesels fell below the strict 8.0 h induction threshold demanded by EN 14214, primarily driven by the significant presence of polyunsaturated fatty acids (such as C18:3). This represents a well-known bottleneck for microalgae biofuels, mandating the downstream addition of synthetic antioxidant formulations (e.g., tert-butylhydroquinone) before commercial commercialization.

These fluctuating fuel properties have direct engineering implications when introduced into modern compression ignition engines. As thoroughly analyzed by Milojević et al. [22], deviations in fuel density and viscosity directly affect fuel injection atomization and spray penetration, which heavily dictate thermal efficiency and exhaust soot/NO_x emissions. For instance, the lower cetane number and degraded nature of the summer batch would cause an extended ignition delay, potentially resulting in incomplete combustion, higher acoustic noise, and elevated emissions, whereas the winter and spring

FAME properties align well with operational parameters required for stable green logistics fleet operations.

3.8. Industrial Implications, Process Optimization, and Circular Bioeconomy Integration

The integration of microalgae cultivation within a pig-slurry biogas plant establishes a highly symbiotic framework for the circular bioeconomy. From an environmental standpoint, this co-location provides two major industrial advantages: it utilizes nutrient-heavy digestate wastewater as a cost-free nutrient source, eliminating the need for expensive synthetic media, while simultaneously sequestering residual CO₂ from flue gases.

What distinguishes this study from conventional literature is the evaluation of these dynamics under real-scale, severe continental climatic constraints (Soria, Spain). The rigorous seasonal characterization provides actionable insights for industrial process optimization. Rather than assuming uniform year-round productivity, operators can utilize these findings to implement dynamic harvesting strategies, such as prioritizing winter biomass (which unexpectedly achieved the highest lipid accumulation and superior FAME profiles due to cold-stress adaptations) for high-quality biodiesel extraction. Conversely, during summer—where intense evaporation and heat stress altered biomass quality—the harvested yield can be strategically redirected to anaerobic co-digestion to boost methane production within the same facility. This flexible biorefinery approach maximizes seasonal resources, proving that decentralized bioenergy plants can remain viable and resilient even under highly fluctuating and harsh environmental conditions.

4. Conclusions

This study demonstrates that seasonal conditions exert a decisive influence on the productivity, lipid accumulation, and fuel-related composition of native microalgae (*Chlorella* sp.) cultivated in an open raceway pond integrated into a pig-slurry biogas plant.

Winter proved to be the most favorable season for biofuel production, yielding the highest lipid accumulation (18.9 wt.% on a dry basis and a volumetric production of 1.47 g · L⁻¹) with a crude ester content of 60.8 wt.% that reached 93.3 wt.% after column chromatography purification. Conversely, summer samples showed the poorest performance due to drought and pink bacteria proliferation. Biomass processing also revealed a strong dependence on post-harvest drying; stable oven-drying at 45 °C maintained concentrations between 7.80 and 8.80 g · L⁻¹, whereas extended outdoor air-drying during spring and summer triggered microbial degradation, dropping biomass densities to 2.86 and 4.33 g · L⁻¹, respectively. Elemental characterization confirmed that the residual biomass has restricted direct applications as a solid fuel due to low heating values (<10MJ · kg⁻¹) and an exceptionally high ash content that rose from 15.98–41.14 wt.% to 41.14–51.91 wt.% after oil extraction. Nevertheless, the predominant C₁₆–C₁₈ FAME profiles confirm a high potential for seasonal biodiesel optimization.

In conclusion, this study demonstrates that seasonal fluctuations in continental climates exert critical control on both microalgae culture stability and the resulting biodiesel properties. To transition these findings from an experimental biorefinery scheme to a fully commercial scale, future research lines must be established. While beyond the scope of this work, subsequent investigations should focus on developing advanced microbiological control protocols to mitigate summer bacterial overgrowth, as well as conducting comprehensive Techno-Economic Analyses (TEAs) and Life-Cycle Assessments (LCAs) to validate the economic and environmental sustainability of the integrated pig-slurry framework. Furthermore, performing physical engine performance and exhaust emission testing utilizing the seasonal FAME blends produced will be essential to confirm their practical applicability in green logistics fleets.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/en19143418/s1>, Figure S1: Oven drying process of microalgae; Figure S2: (a), (b) Compact microalgae after oven drying (c) microalgae after crushing; Figure S3: Chromatographic column to purify the FAMES; Figure S4: FT-IR spectrum of spring oil (transmittance vs. wavenumber); Figure S5: FT-IR spectrum of spring FAME (transmittance vs. wavenumber); Figure S6: UV-VIS spectrum of spring oil; Figure S7: UV-VIS spectrum of spring FAMES; Table S1: Reagents used in the experiments.

Author Contributions: A.R., data curation, investigation, writing—original draft. I.T., investigation, methodology, supervision. D.B., methodology, supervision, validation, reviewing and editing. B.C., data curation, investigation, validation, reviewing and editing. M.L., funding, resources, project administration, reviewing and editing. L.C., conceptualization, validation, funding, methodology, writing—original draft, reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the following projects. (1) REQWATER “Improving energy production and the circularity of anaerobic digestion by integrating advanced oxidation and electrochemical processes into the treatment strategy”, Grant PID 2023-152563OB-I00, funded by the Ministry of Science, Innovation and Universities of Spain/Agencia Estatal de Investigación/10.13039/501100011033 and FEDER, EU. (2) AQUAHUB “Promoting water quality, sustainability and the circular economy through a Madrid-based hub for innovative technological solutions”, with reference number 59/561770.9/24, funded under the 2024 Call for Applications for grants aimed at improving public–private collaboration in the field of R&D&I through consortium-based flagship projects, co-financed by the European Regional Development Fund FEDER under the Community of Madrid’s Operational Program for the period 2021–2027.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Acknowledgments: The authors wish to thank José Eugenio Ortíz of UPM for the GC-MS spectra, Lucía Arregui and Belén Patiño from the Universidad Complutense de Madrid for the microscopy of microalgae, Francisco Sanz from Repsol for the GC-GC-TOF-MS spectra, and the UPM technical staff Jerónimo-Emilio, García-González, and Cristina Fonollá for their help in the laboratory. During the preparation of this work, the authors used ChatGPT 5.5 to improve writing, readability, and English language quality. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

Conflicts of Interest: The authors declare no competing interests.

References

1. EU 2018/2001; Directive (EU) 2018/2001 of the European Parliament and of the Council of 11 December 2018 on the Promotion of the Use of Energy from Renewable Sources (Recast). European Parliament and the Council of the European Union: Strasbourg, France, 2018.
2. EU 2023/2413; Directive (EU) 2023/2413 of the European Parliament and of the Council of 18 October 2023 Amending Directive (EU) 2018/2001, Regulation (EU) 2018/1999 and Directive 98/70/EC as Regards the Promotion of Energy from Renewable Sources, and Repealing Council Directive (EU) 2015/652. European Parliament and the Council of the European Union: Strasbourg, France, 2023.
3. Chisti, Y. Biodiesel from Microalgae. *Biotechnol. Adv.* **2007**, *25*, 294–306. [[CrossRef](#)] [[PubMed](#)]
4. Milano, J.; Ong, H.C.; Masjuki, H.H.; Chong, W.T.; Lam, M.K.; Loh, P.K.; Vellayan, V. Microalgae Biofuels as an Alternative to Fossil Fuel for Power Generation. *Renew. Sustain. Energy Rev.* **2016**, *58*, 180–197. [[CrossRef](#)]
5. Tobío-Pérez, I.; Alfonso-Cardero, A.; Díaz-Domínguez, Y.; Pohl, S.; Piloto-Rodríguez, R.; Lapuerta, M. Thermochemical Conversion of *Sargassum* for Energy Production: A Comprehensive Review. *BioEnergy Res.* **2022**, *15*, 1872–1893. [[CrossRef](#)]
6. Sánchez-Borroto, Y.; Lapuerta, M.; Melo-Espinosa, E.A.; Bolonio, D.; Tobío-Pérez, I.; Piloto-Rodríguez, R. Green-Filamentous Macroalgae *Chaetomorpha cf. gracilis* from Cuban Wetlands as a Feedstock to Produce Alternative Fuel: A Physicochemical Characterization. *Energy Sources Part A Recovery Util. Environ. Eff.* **2018**, *40*, 1279–1289. [[CrossRef](#)]

7. Razzak, S.A.; Lucky, R.A.; Hossain, M.M.; deLasa, H. Valorization of Microalgae Biomass to Biofuel Production: A Review. *Energy Nexus* **2022**, *7*, 100139. [\[CrossRef\]](#)
8. Gaurav, K.; Neeti, K.; Singh, R. Microalgae-Based Biodiesel Production and Its Challenges and Future Opportunities: A Review. *Green Technol. Sustain.* **2024**, *2*, 100060. [\[CrossRef\]](#)
9. Jha, S.; Singh, R.; Pandey, B.K.; Tiwari, A.K.; Shukla, S.; Dikshit, A.; Bhardwaj, A.K. Recent Aspects of Algal Biomass for Sustainable Fuel Production: A Review. *Discov. Sustain.* **2024**, *5*, 300. [\[CrossRef\]](#)
10. Hoang, A.T.; Sirohi, R.; Pandey, A.; Nižetić, S.; Lam, S.S.; Chen, W.-H.; Luque, R.; Thomas, S.; Arıcı, M.; Pham, V.V. Biofuel Production from Microalgae: Challenges and Chances. *Phytochem. Rev.* **2023**, *22*, 1089–1126. [\[CrossRef\]](#)
11. Tobío-Pérez, I.; Bolonio, D.; Ortega, M.F.; De Godos, I.; Lapuerta, M.; Canoira, L. Sustainable Biofuel from Microalgae Cultivated with Waste Streams from a Biogas Plant. *Discov. Energy* **2026**, *6*, 31. [\[CrossRef\]](#)
12. Tang, D.; Han, W.; Li, P.; Miao, X.; Zhong, J. CO₂ Biofixation and Fatty Acid Composition of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in Response to Different CO₂ Levels. *Bioresour. Technol.* **2011**, *102*, 3071–3076. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Torres-Martínez, J.J.; Martínez-Constantino, A.; González-Ortega, O.; Dibildox-Alvarado, E.; Soria-Guerra, R.E. Impact of Blue and Red Light on Lipid Accumulation in *Chlorella vulgaris* and *Scenedesmus acutus*. *J. Appl. Phycol.* **2025**, *37*, 2301–2311. [\[CrossRef\]](#)
14. Ahmed, A.S.; Ali, A.; Gorgun, E.; Jameel, M.; Khandaker, T.; Islam, M.S.; Islam, M.S.; Abdullah, M. Microalgae to Biofuel: Cutting-Edge Harvesting and Extraction Methods for Sustainable Energy Solution. *Energy Sci. Eng.* **2025**, *13*, 3525–3540. [\[CrossRef\]](#)
15. Zhou, C.; Yuan, M.; Huang, C.; Chen, Q.; Wang, J.; Chen, X.; Yang, H.; Fang, J.; Yang, B. Enhancing Biomass Production of *Chlorella vulgaris* in Anaerobically Digested Swine Wastewater Using Carbon Supplementation and Simultaneous Lipid Production. *Appl. Sci.* **2025**, *15*, 5103. [\[CrossRef\]](#)
16. Bhattacharya, S.; Coelho, R.S.; Franco, T.T.; Zuorro, A.; Vyas, A.; Mukherjee, T.; Joshi, V.C.; Trif, M. Conversion of Pentose Sugars from Lignocellulosic Hydrolysate to Isopropyl Alcohol via Integrated *Chlorella vulgaris* Cultivation and Yeast Fermentation: An Alternative Fuel to Diesel Engines. *Algal Res.* **2025**, *87*, 103982. [\[CrossRef\]](#)
17. Mandal, S.; Mallick, N. Microalga *Scenedesmus obliquus* as a Potential Source for Biodiesel Production. *Appl. Microbiol. Biotechnol.* **2009**, *84*, 281–291. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Schomaker, R.A.; Dudycha, J.L. De Novo Transcriptome Assembly of the Green Alga *Ankistrodesmus falcatus*. *PLoS ONE* **2021**, *16*, e0251668. [\[CrossRef\]](#) [\[PubMed\]](#)
19. George, B.; Pancha, I.; Desai, C.; Chokshi, K.; Paliwal, C.; Ghosh, T.; Mishra, S. Effects of Different Media Composition, Light Intensity and Photoperiod on Morphology and Physiology of Freshwater Microalgae *Ankistrodesmus falcatus*—A Potential Strain for Bio-Fuel Production. *Bioresour. Technol.* **2014**, *171*, 367–374. [\[CrossRef\]](#) [\[PubMed\]](#)
20. Gu, D.; Xiao, Q.; Zhao, Y.; Yu, X. A Low-Cost Technique for Biodiesel Production in *Ankistrodesmus* Sp. EHY by Using Harvested Microalgal Effluent. *Sci. Total Environ.* **2023**, *857*, 159461. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Yee, W. Microalgae from the Selenastraceae as Emerging Candidates for Biodiesel Production: A Mini Review. *World J. Microbiol. Biotechnol.* **2016**, *32*, 64. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Milojević, S.; Stopka, O.; Kontrec, N.; Orynycz, O.; Hlatká, M.; Radojković, M.; Stojanović, B. Analytical Characterization of Thermal Efficiency and Emissions from a Diesel Engine Using Diesel and Biodiesel and Its Significance for Logistics Management. *Processes* **2025**, *13*, 2124. [\[CrossRef\]](#)
23. Konga, A.K.; Muchandi, A.S.; Ponnaiah, G.P. Soxhlet Extraction of *Spirogyra* Sp. Algae: An Alternative Fuel. *Biofuels* **2017**, *8*, 29–35. [\[CrossRef\]](#)
24. Zhou, J.; Wang, M.; Saraiva, J.A.; Martins, A.P.; Pinto, C.A.; Prieto, M.A.; Simal-Gandara, J.; Cao, H.; Xiao, J.; Barba, F.J. Extraction of Lipids from Microalgae Using Classical and Innovative Approaches. *Food Chem.* **2022**, *384*, 132236. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Boni, J.; Aida, S.; Leila, K. Lipid Extraction Method from Microalgae *Botryococcus braunii* as Raw Material to Make Biodiesel with Soxhlet Extraction. *J. Phys. Conf. Ser.* **2018**, *1095*, 012004. [\[CrossRef\]](#)
26. Halim, R.; Gladman, B.; Danquah, M.K.; Webley, P.A. Oil Extraction from Microalgae for Biodiesel Production. *Bioresour. Technol.* **2011**, *102*, 178–185. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Canoira, L.; Rodríguez-Gamero, M.; Querol, E.; Alcántara, R.; Lapuerta, M.; Oliva, F. Biodiesel from Low-Grade Animal Fat: Production Process Assessment and Biodiesel Properties Characterization. *Ind. Eng. Chem. Res.* **2008**, *47*, 7997–8004. [\[CrossRef\]](#)
28. Alcántara, R.; Amores, J.; Canoira, L.; Fidalgo, E.; Franco, M.J.; Navarro, A. Catalytic Production of Biodiesel from Soy-Bean Oil, Used Frying Oil and Tallow. *Biomass Bioenergy* **2000**, *18*, 515–527. [\[CrossRef\]](#)
29. Donoso, D.; Bolonio, D.; Ballesteros, R.; Lapuerta, M.; Canoira, L. Hydrogenated Orange Oil: A Waste Derived Drop-in Biojet Fuel. *Renew. Energy* **2022**, *188*, 1049–1058. [\[CrossRef\]](#)
30. Lapuerta, M.; Hernández, J.J.; Rodríguez, J. Kinetics of Devolatilisation of Forestry Wastes from Thermogravimetric Analysis. *Biomass Bioenergy* **2004**, *27*, 385–391. [\[CrossRef\]](#)

31. Ortega, M.F.; Donoso, D.; Bousbaa, H.; Bolonio, D.; Ballesteros, R.; García-Martínez, M.-J.; Lapuerta, M.; Canoira, L. Optimized Production of Fatty Acid Ethyl Esters (FAEE) from Waste Frying Oil by Response Surface Methodology. *Waste Biomass Valor.* **2021**, *12*, 2303–2310. [[CrossRef](#)] [[PubMed](#)]
32. Jeong, S.W.; Yang, J.E.; Choi, Y.J. Isolation and Characterization of a Yellow Xanthophyll Pigment-Producing Marine Bacterium, *Erythrobacter* Sp. SDW2 Strain, in Coastal Seawater. *Mar. Drugs* **2022**, *20*, 73. [[CrossRef](#)] [[PubMed](#)]
33. Domenici, V.; Ancora, D.; Cifelli, M.; Serani, A.; Veracini, C.A.; Zandomeneghi, M. Extraction of Pigment Information from Near-UV Vis Absorption Spectra of Extra Virgin Olive Oils. *J. Agric. Food Chem.* **2014**, *62*, 9317–9325. [[CrossRef](#)] [[PubMed](#)]
34. Erdoğan, A.; Çağır, A.; Dalay, M.C.; Eroğlu, A.E. Composition of Carotenoids in *Scenedesmus protuberans*: Application of Chromatographic and Spectroscopic Methods. *Food Anal. Methods* **2015**, *8*, 1970–1978. [[CrossRef](#)]
35. Abomohra, A.E.-F.; El-Sheekh, M.; Hanelt, D. Pilot Cultivation of the Chlorophyte Microalga *Scenedesmus obliquus* as a Promising Feedstock for Biofuel. *Biomass Bioenergy* **2014**, *64*, 237–244. [[CrossRef](#)]
36. Beltrán-Rocha, J.C.; Guajardo-Barbosa, C.; Barceló-Quintal, I.D.; López-Chuken, U.J. Biotratamiento de Efluentes Secundarios Municipales Utilizando Microalgas: Efecto Del pH, Nutrientes (C, N y P) y Enriquecimiento Con CO₂. *Rev. Biol. Mar. Oceanogr.* **2017**, *52*, 417–427. [[CrossRef](#)]
37. Abdullah, N.; Amran, N.A.; Yasin, N.H. Algae Oil Extraction from Freshwater Microalgae *Chlorella vulgaris*. *Malays. J. Anal. Sci.* **2017**, *21*, 735–744. [[CrossRef](#)]
38. Mat Yasin, N.H.; Mohd Yusop, M.A.; Awang Kechik, N.A.A.; Zahuri, A.Z. Composition of Fatty Acid Methyl Ester in Microalgae *Chlorella vulgaris*: Comparison between Various Methods of Harvesting, Extraction and Transesterification. *Mater. Today Proc.* **2023**, S2214785323022186. [[CrossRef](#)]
39. Mubarak, M.; Shaija, A.; Suchithra, T.V. A Review on the Extraction of Lipid from Microalgae for Biodiesel Production. *Algal Res.* **2015**, *7*, 117–123. [[CrossRef](#)]
40. Yin, C.-Y. Prediction of Higher Heating Values of Biomass from Proximate and Ultimate Analyses. *Fuel* **2011**, *90*, 1128–1132. [[CrossRef](#)]
41. Nhuchhen, D.R.; Abdul Salam, P. Estimation of Higher Heating Value of Biomass from Proximate Analysis: A New Approach. *Fuel* **2012**, *99*, 55–63. [[CrossRef](#)]
42. Nishshanka, G.K.S.H.; Liyanaarachchi, V.C.; Nimarshana, P.H.V.; Ariyadasa, T.U.; Chang, J.-S. *Haematococcus pluvialis*: A Potential Feedstock for Multiple-Product Biorefining. *J. Clean. Prod.* **2022**, *344*, 131103. [[CrossRef](#)]
43. Magalhães, I.B.; Pereira, A.S.A.D.P.; Silva, T.A.; Renato, N.D.S. Predicting the Higher Heating Value of Microalgae Biomass Based on Proximate and Ultimate Analysis. *Algal Res.* **2022**, *64*, 102677. [[CrossRef](#)]
44. Kumar, K.; Dasgupta, C.N.; Das, D. Cell Growth Kinetics of *Chlorella sorokiniana* and Nutritional Values of Its Biomass. *Bioresour. Technol.* **2014**, *167*, 358–366. [[CrossRef](#)] [[PubMed](#)]
45. Jabeen, S.; Gao, X.; Altarawneh, M.; Hayashi, J.; Zhang, M.; Dlugogorski, B.Z. Analytical Procedure for Proximate Analysis of Algal Biomass: Case Study for *Spirulina platensis* and *Chlorella vulgaris*. *Energy Fuels* **2020**, *34*, 474–482. [[CrossRef](#)]
46. Senila, L.; Kovacs, E.; Roman, C. Chemical Characterization, Lipid Profile, and Volatile Compounds in *Chlorella* Sp. and *Spirulina platensis*: A Promising Feedstock for Various Applications. *Molecules* **2025**, *30*, 1499. [[CrossRef](#)] [[PubMed](#)]
47. Ross, A.; Jones, J.; Kubacki, M.; Bridgeman, T. Classification of Macroalgae as Fuel and Its Thermochemical Behaviour. *Bioresour. Technol.* **2008**, *99*, 6494–6504. [[CrossRef](#)] [[PubMed](#)]
48. Dutta, R.; Sarkar, U.; Mukherjee, A. Extraction of Oil from *Crotalaria juncea* Seeds in a Modified Soxhlet Apparatus: Physical and Chemical Characterization of a Prospective Bio-Fuel. *Fuel* **2014**, *116*, 794–802. [[CrossRef](#)]
49. Alves, J.L.F.; Da Silva, J.C.G.; Costa, R.L.; Dos Santos Junior, S.F.; Da Silva Filho, V.F.; Moreira, R.D.F.P.M.; José, H.J. Investigation of the Bioenergy Potential of Microalgae *Scenedesmus acuminatus* by Physicochemical Characterization and Kinetic Analysis of Pyrolysis. *J. Therm. Anal. Calorim.* **2019**, *135*, 3269–3280. [[CrossRef](#)]
50. Mustapha, S.I.; Mohammed, U.A.; Rawat, I.; Bux, F.; Isa, Y.M. Production of High-Quality Pyrolytic Bio-Oils from Nutrient-Stressed *Scenedesmus obliquus* Microalgae. *Fuel* **2023**, *332*, 126299. [[CrossRef](#)]
51. Zhang, B.; Lin, Q.; Zhang, Q.; Wu, K.; Pu, W.; Yang, M.; Wu, Y. Catalytic Hydrothermal Liquefaction of *Euglena* Sp. Microalgae over Zeolite Catalysts for the Production of Bio-Oil. *RSC Adv.* **2017**, *7*, 8944–8951. [[CrossRef](#)]
52. Gong, Z.; Fang, P.; Wang, Z.; Li, X.; Wang, Z.; Meng, F. Pyrolysis Characteristics and Products Distribution of *Haematococcus pluvialis* Microalgae and Its Extraction Residue. *Renew. Energy* **2020**, *146*, 2134–2141. [[CrossRef](#)]
53. Sheng, C.; Azevedo, J.L.T. Estimating the Higher Heating Value of Biomass Fuels from Basic Analysis Data. *Biomass Bioenergy* **2005**, *28*, 499–507. [[CrossRef](#)]
54. Channiwala, S.A.; Parikh, P.P. A Unified Correlation for Estimating HHV of Solid, Liquid and Gaseous Fuels. *Fuel* **2002**, *81*, 1051–1063. [[CrossRef](#)]
55. Grace, C.E.E.; Lakshmi, P.K.; Meenakshi, S.; Vaidyanathan, S.; Srisudha, S.; Briget Mary, M. Biomolecular Transitions and Lipid Accumulation in Green Microalgae Monitored by FTIR and Raman Analysis. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2020**, *224*, 117382. [[CrossRef](#)] [[PubMed](#)]

56. Patel, A.K.; Albarico, F.P.J.B.; Perumal, P.K.; Vadrle, A.P.; Nian, C.T.; Chau, H.T.B.; Anwar, C.; Wani, H.M.U.D.; Pal, A.; Saini, R.; et al. Algae as an Emerging Source of Bioactive Pigments. *Bioresour. Technol.* **2022**, *351*, 126910. [[CrossRef](#)] [[PubMed](#)]
57. Vladoic, J.; Radman, S.; Zizak, Z.; Besu, I.; Jerkovic, I.; Speranza, L.G.; Hala, A.F.; Kovacevic, S.; Perreira, H.; Gouveia, L. Clean Production of Microalgae High-Value Lipid Fraction: Influence of Different Pretreatments on Chemical and Cytotoxic Profiles of *Chlorella vulgaris* Supercritical Extracts and Life Cycle Assessment. *J. Clean. Prod.* **2025**, *491*, 144823. [[CrossRef](#)]
58. Giridhar Babu, A.; Wu, X.; Kabra, A.N.; Kim, D.-P. Cultivation of an Indigenous *Chlorella sorokiniana* with Phytohormones for Biomass and Lipid Production under N-Limitation. *Algal Res.* **2017**, *23*, 178–185. [[CrossRef](#)]
59. Holtin, K.; Kuehnle, M.; Rehbein, J.; Schuler, P.; Nicholson, G.; Albert, K. Determination of Astaxanthin and Astaxanthin Esters in the Microalgae *Haematococcus pluvialis* by LC-(APCI)MS and Characterization of Predominant Carotenoid Isomers by NMR Spectroscopy. *Anal. Bioanal. Chem.* **2009**, *395*, 1613–1622. [[CrossRef](#)] [[PubMed](#)]
60. Damiani, M.C.; Popovich, C.A.; Constenla, D.; Leonardi, P.I. Lipid Analysis in *Haematococcus pluvialis* to Assess Its Potential Use as a Biodiesel Feedstock. *Bioresour. Technol.* **2010**, *101*, 3801–3807. [[CrossRef](#)] [[PubMed](#)]
61. Kings, A.J.; Raj, R.E.; Miriam, L.R.M.; Visvanathan, M.A. Cultivation, Extraction and Optimization of Biodiesel Production from Potential Microalgae *Euglena sanguinea* Using Eco-Friendly Natural Catalyst. *Energy Convers. Manag.* **2017**, *141*, 224–235. [[CrossRef](#)]
62. Bolonio, D.; Llamas, A.; Rodríguez-Fernández, J.; Al-Lal, A.M.; Canoira, L.; Lapuerta, M.; Gómez, L. Estimation of Cold Flow Performance and Oxidation Stability of Fatty Acid Ethyl Esters from Lipids Obtained from *Escherichia coli*. *Energy Fuels* **2015**, *29*, 2493–2502. [[CrossRef](#)]
63. Houachri, T.; Bolonio, D.; Llamas, A.; Rodríguez-Fernández, J.; El Gazzah, M.; Mittelbach, M.; Lapuerta, M.; Canoira, L. Fatty Acid Methyl and Ethyl Esters Obtained from Rare Seeds from Tunisia: *Ammi visnaga*, *Citrullus colocynthis*, *Datura stramonium*, *Ecballium elaterium*, and *Silybum marianum*. *Energy Sources Part A Recovery Util. Environ. Eff.* **2018**, *40*, 93–99. [[CrossRef](#)]
64. Houachri, T.; Bolonio, D.; Llamas, A.; Rodríguez-Fernández, J.; El Gazza, M.; Mittelbach, M.; Lapuerta, M.; Canoira, L. Fatty Acid Methyl Esters (FAME) from Oleaginous Seeds Grown in Arid Lands. Part II: *Ibicella lutea*, *Onopordum nervosum*, *Peganum harmala*, *Smyrniolum olusatrum* and *Solanum elaeagnifolium*. *Energy Sources Part A Recovery Util. Environ. Eff.* **2018**, *40*, 1434–1441. [[CrossRef](#)]
65. EN 14214:2012+A2:2019; Liquid Petroleum Products—Fatty Acid Methyl Esters (FAME) for Use in Diesel Engines and Heating Applications—Requirements and Test Methods. European Committee for Standardization (CEN) CEN: Brussels, Belgium, 2019.

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.