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Growth Performance and Biochemical Composition of Waste-Isolated Microalgae Consortia Grown on Nano-Filtered Pig Slurry and Cheese Whey under Mixotrophic Conditions

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Abstract: The cultivation mode plays a vital role in algal growth and composition. This paper assessed the growth ability of twelve algae–microbial consortia (ACs) originally selected from organic wastes when nano-filtered pig slurry wastewater (NFP) and cheese whey (CW) were used as growth substrates in a mixotrophic mode in comparison with a photoautotrophic mode. Nutrient uptake ability, biochemical composition, fatty acids, and amino acid profiles of ACs were compared between both cultivation conditions. On average, 47% higher growth rates and 35% higher N uptake were found in mixotrophic cultivation along with significant P and TOC removal rates. Changing the cultivation mode did not affect AA and FA composition but improved EAA content, providing the potential for AC_5 and AC_4 to be used as local protein feed supplements. The results also showed the possibility for AC_6 and AC_1 to be used as omega-3 supplements due to their low ω-6–ω-3 ratio.

Keywords: biochemical composition; cheese whey; microalgae consortium; mixotrophic cultivation; nutrient assimilation

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1. Introduction

Microalgae cultivation has been carried out worldwide to produce animal feed or high-value products, along with wastewater treatment, carbon dioxide (CO_2) mitigation, or as a feedstock for biofuel production [1]. Microalgae can be cultivated both autotrophically and mixotrophically. Nonetheless, the photoautotrophic mode presents several limitations including low cell densities and long cultivation periods [2]. Mixotrophic cultivation, in which organic carbon (C), light energy, and CO_2 are simultaneously supplied to the algae in the system, has been proposed as a potential mode for algae biomass and lipid production [3], where the growth of microalgae does not entirely depend on photosynthesis. Under mixotrophic conditions, microalgae grow both photoautotrophically and heterotrophically since they are able to assimilate organic compounds as a carbon source and use inorganic carbon as an electron donor [4]. In this type of metabolism, the energy is captured through the catalysis of external organic compounds through respiration, and the light energy is converted into chemical compounds via photosynthesis. Studies have shown that some microalgae grow rapidly and have higher growth rates and biomass yields under mixotrophic conditions than under autotrophic conditions [2,5].

However, the cost of the organic carbon sources is high when compared to all other added nutrients, being about 80% of the total cost of the cultivation medium [6]. To overcome this high carbon cost, less costly organic sources are required [7]. Cheese whey (CW), which is the liquid by-product remaining from the cheese manufacturing process and contains high organic matter content, can provide such a supply.

The Lombardy region produces large amounts of CW each year; for example, in 2019 the region produced around 3692 tons of cheese whey, which represented 35.4% of total Italian CW production [8]. Moreover, the Italian dairy industry has been of great

concern due to its high CO_2 emissions, mainly due to cattle farming. Utilizing CW for algae growth can reduce the environmental pressure, leading to waste valorization through the production of value-added bioproducts.

The other important and necessary nutrients for microalgae growth are nitrogen (N) and phosphorus (P). High costs are also involved in supplying these nutrients, for instance, in producing 100 tons of microalgae biomass, up to 10 tons of N and 1 ton of P are consumed [9]. In the quest for inexpensive algal biotechnology, the combining of wastewater treatment and microalgal cultivation has been promoted as a viable means of reducing the nutrient cost as well as the freshwater input [10]. Microalgae-based processes can reduce by up to half the energy consumption of conventional wastewater treatment and allow the recovery of up to 90% of the nutrients contained in wastewater [11]. Nano-filtered pig slurry permeate (NFP) is the effluent of a pig slurry treatment factory, which contains a high percentage of ammonium (NH_3-N) in low concentrations and could be considered a suitable nitrogen source to support algal growth [12] since algae prefer ammonium N as the N source.

In recent years, intensive investigation into the biological removal of carbon, nitrogen, and phosphorus via microalgae in waste and wastewater effluents has been evaluated by several studies. It has been performed with various microalgae species on a range of wastewater types, including municipal, agricultural, brewery, textile, and industrial effluents, with promising results in treatment performance and microalgae growth [13–16]. *Scenedesmus obliquus* has been demonstrated to successfully remove nutrients from municipal wastewater with ammonia nitrogen and total phosphorus removed at up to 100% removal efficiencies [13]. The maximum removal of ammonia nitrogen, chemical oxygen demand, total nitrogen, and total phosphorus reached 94.38%, 88.52%, 96.71%, and 98.68% for *Chlorella* sp. grown in brewery wastewater, reported by Wang et al. [16].

Thus, the use of agro-industrial by-products stands out for its potential, adding value to production processes and reducing costs. The nutritional characteristics, availability, and low cost give the possibility of using the by-products in the cultivation of microalgae, along with high nutrient removal efficiencies. However, certain limitations remain to be resolved when combining microalgae cultivation with the two waste streams of interest, i.e., NFP and CW. They might contain several compounds at elevated concentrations as well as unwanted organisms that inhibit biological (e.g., microalgal) growth, especially for pure algae species. Pure microalgae cultivation in organic wastes may be hampered by their low adaptation to extreme growth conditions and by the risk of microbial contamination [17]. As a solution, microalgae consortia cultures can be used due to their high resistance to external factors such as unwanted and detrimental species. Moreover, microalgae consortia are efficient in detoxifying organic and inorganic pollutants, and removing nutrients from wastewater, compared to the individual microorganisms [18].

Recently (in 2020) the Lombardy region (North Italy) (see Founding section), funded a broader project aimed at isolating and studying indigenous, autochthonous algae–microbial consortia (ACs) from various local organic wastes with different origins to be used for technological applications to treat organic wastes, remove nutrients, and produce useful biomass both in the lab and at pilot scale. The first part of this project has already been carried out. In it, twelve algae consortia (ACs) were isolated from sixteen different organic wastes [17]. Then the ACs were tested to treat organic wastes, removing nutrients and producing useful biomass in different modes, i.e., autotrophic mode, as previously reported [12], and mixotrophic mode (this paper), at batch scale, before up-scaling to a pilot plant.

The innovation of the approach is to exploit the potential of local ACs isolated from local wastes to be used to treat local wastes, creating an approach to be replicated elsewhere.

Therefore, this study aimed to combine an ammonium nitrogen-rich waste stream, i.e., NFP, and an organic carbon-rich waste stream, i.e., CW, to locally promote the production of by-products by using twelve different microalgae consortia previously selected [13] under mixotrophic conditions, and previously tested under the autotrophic mode [12]. The

growth performance, nutrient recovery ability, and biochemical composition, including profiles of fatty acids and amino acids in the mixotrophic mode were evaluated and compared with the results from autotrophic conditions. This work aims to investigate whether changing the cultivation mode affects the growth performance and biomass quality of algae consortia.

2. Materials and Methods

2.1. Nano-Filtered Permeate and Cheese Whey Sampling and Characterization

The culture medium used during batch trials was the nano-filtered permeate (NFP) sampled at a full-scale pig slurry treatment plant located in Brescia province in Northern Italy. In brief, the pig slurry is subject to a two-step solid/liquid separation obtaining a liquid fraction (on average, 1.5 ± 2% of TS and 2103 ± mg kg^{-1} TKN; $NH4^+$/TKN of 96%) that is successively treated by nano-filtration (obtaining NFP) and reverse osmosis (operated at full-scale plant) producing a concentrated ammonia (Renure) to be used in agriculture as fertilizer, and clean water, that is discharged directly to surface water bodies.

Cheese whey liquid dairy waste (here called cheese whey, CW) was provided by Caseificio Battipaglia Freschi Minaci s.r.l., a dairy factory located in Brugherio (MB, Lombardy, Italy). After collection, the CW was stored at −20 °C.

NFP and CW were immediately characterized after sampling. Total nitrogen (TN), ammonia nitrogen (NH_4^+-N), pH, and chemical oxygen demand (COD) were determined on fresh materials according to the analytical methods for wastewater sludge [19]. Macro and microelement contents, including Na, Mg, K, Ca, P, Mn, Fe, Cu, Zn, Cr, Co, Ni, As, Se, Mo, Cd, Pb, were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Aurora M90 Bruker, Bremen, Germany), preceded by microwave-assisted (Multiwave ECO, Anton Paar GmbH, Ost-fildern, Germany) nitric acid digestion of fresh samples. All analyses were performed in triplicate.

2.2. Microalgae Consortia and Preparation of Inoculum

The algae consortia used in trials were obtained previously by their direct isolation from organic wastes sampled directly at full-scale plants located in Northern Italy [17]. The aim was to select algae–microbial consortia well adapted to substrates both rich in C and having high nutrient contents. The isolated algae consortia (AC) were maintained in 500 mL Erlenmeyer flasks in BG-11 medium under constant aeration and mixed by using filtered air (filter of 0.2 μm) with a continuous illumination of 50 μE m^{-2} s^{-1}, provided by fluorescent white tubes, at a controlled temperature of 22 ± 1 °C. The twelve algae consortia isolated from the sixteen organic wastes [17] were subject to metagenomic characterization by Next Generation Sequencing (NGS) analysis [17].

2.3. Microalgae Consortia Molecular Characterization

Next Generation Sequencing (NGS) analysis indicated that ACs were characterized by the presence of both eukaryotes and prokaryotes. According to previous work [17], they were classified as the following: *Chlorella*-dominated consortium (AC_1), *Tetradesmus*-dominated consortium (AC_6), *Chlorella* and *Synechocystis* (Cyanobacteria)-co-dominated consortia (AC_2, AC_3, AC_4, AC_5, AC_9, and AC_11), *Tetradesmus* and *Synechocystis*-co-dominated consortia (AC_7 and AC_10), *Chlorella* and *Tetradesmus*-co-dominated consortium (AC_12) and, finally, a low algae presence consortium (AC_8).

2.4. Experimental Set-Up and Cultivation

This study aimed to assess growth performance and chemical and biological characteristics of algae consortia previously isolated from organic wastes, using two combined waste streams, i.e., pig slurry-derived product (NFP) and cheese whey (CW), as the culture medium (see Section 2.1).

To do so, batch trials were carried out in triplicate in self-built photo-bioreactors (PBRs). These were composed of glass vessels of 0.5 L working volume, supplied with

air through plastic pipes connected to an air compressor equipped with a flowmeter and supplied with CO_2 through a CO_2 cylinder. pH was set at 8 and was maintained by using a pure CO_2 injection adopting an "on-demand" modality [20]. Room temperature (25 °C) and constant air flux (10 L min^{-1}) were provided as well as the light that was delivered by cold fluorescent lamps at an irradiance of 312 µE m^{-2} s^{-1} at the PBR surface. A 12 h:12 h photoperiod regime was selected, as proposed in previous works for lab culturing of both pure microalgae and microalgae consortia. This provides the light regime suitable for almost all strains present in the inocula [5,21]. In autotrophic mode, NFP alone was used as the batch growth medium, except for the addition to the culture medium of both P (7.11 mg L^{-1} of K_2HPO_4) and Fe (1.02 mg L^{-1} $(NH_4)_5[Fe(C_6H_4O_7)_2]$) to obtain a complete substrate growth. The final autotrophic culture medium consisted of 90% NFP (v/v) and 10% algae inoculum (v/v). In mixotrophic mode, NFP and CW were used as the batch growth medium with no additional nutritional supplement. The final mixotrophic culture medium consisted of 80% NFP (v/v), 10% CW (v/v), and 10% algae inoculum (v/v). This provided nutrients and kept the ammonium concentration below 150 mg L^{-1} [5] as well as maintaining organic carbon concentration below 0.1 Mol L^{-1} [22], thus avoiding inhibiting conditions.

The microalgae consortia inocula were maintained under the conditions described in Section 2.2 before being used as inoculates for batch cultivation. During the maintenance period, about one third of the culture medium (depending on the growth of each AC) was replaced by sterilized BG-11 nutrient solution twice a week under sterilized environments. This allowed the concentration of microalgae consortia inocula to be kept at between 2 g L^{-1} and 3 g L^{-1}, thus ensuring a biomass concentration in the batch between 0.2 g L^{-1} and 0.3 g L^{-1} [5]. The method used to determine microalgae consortia dry weight (DW) both in inocula and in batch cultures is described in Section 2.5. Further molecular biology analysis needs to be performed to ensure ACs stability and uniformity of inocula, above all, before going from batch to pilot scale plant.

2.5. Microalgae Consortia Growth Determination

Microalgae consortia dry weight (DW) was determined by sampling 5 mL of algae suspension from each PBR. The samples were centrifuged at 4000 rpm for 10 min and then washed with an equivalent volume of distilled water to remove salts. Culture samples were then filtered by a 1.2 µm filter (GF/C, Whatman Ltd., Maidstone, UK) and dried overnight at 80 °C. Sampling was performed every day.

The specific growth rate µ (day^{-1}) was calculated from Equation (1)

$$\mu = (\ln N_1 - \ln N_2)/(t_1 - t_2) \quad (1)$$

where N_1 and N_2 are the concentrations of cells (g L^{-1}) at days t_1 and t_2.

The nitrogen (N) taken up by biomass was calculated according to Equation (2)

$$\text{N taken up by biomass} = (\text{TNbiomass} \times \text{DB})/\text{Ninitial}\% \quad (2)$$

in which $TN_{initial}$ (mg L^{-1}) is the nitrogen concentration at the beginning, TN_{final} (mg L^{-1}) is the nitrogen concentration at the end of the experiment, $TN_{biomass}$ (mg kg^{-1}) is the concentration of TN in the biomass, and DB (kg L^{-1}) is the dry biomass produced per L of growth medium.

The phosphorus (P) taken up by biomass was calculated according to Equation (3) [23]

$$\text{P taken up by biomass} = (\text{Pinitial} - \text{Pfinal})/\text{Pinitial}\% \quad (3)$$

in which $P_{initial}$ (mg L^{-1}) is the phosphorus concentration at the beginning and P_{final} (mg L^{-1}) is the phosphorus concentration at the end of the experiment.

2.6. Biochemical Analysis

The TN concentration ($TN_{biomass}$, g kg^{-1} DM) was detected on biomass samples of about 0.2–0.3 g, using an elementary analyzer (Elementar Rapid max N exceed, Elementar Italia s.r.l., Lomazzo, Italy), based on the analytical method of combustion "Dumas" and equipped with a thermal conductivity detector (TCD, Elementar Italia s.r.l., Lomazzo, Italy). The crude protein contents were estimated by multiplying the total N by the conversion factor of 6.25 [24]. The ash content was determined as the residue after ignition at 550 °C overnight. Carbohydrates were estimated by subtracting the percentage of ashes, lipids, and crude proteins out of 100%. The mass balance was verified as the residual fraction (composed of carbohydrates and soluble non-protein cell content), calculated as the missing part of the total weight, as previously proposed [25].

The amino acid (AA) content of algal consortia biomass was determined by the HPLC-DAD technique with some modifications [24]. Acidic hydrolysis of samples was used for the determination of lysine (Lys), histidine (His), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), valine (Val), threonine (Thr), arginine (Arg), alanine (Ala), glycine (Gly), proline (Pro), glutamic acid (Glu), serine (Ser), aspartic acid (Asp), and tyrosine (Tyr). About 0.1–0.2 g of freeze-dried samples were hydrolyzed in 10 mL of 6 Mol L^{-1} HCl for 24 h in a water bath at 100 °C, followed by neutralization with NaOH. For the determination of sulfur amino acids (Met and Cys), the samples were pretreated with 1 mL of a mixture of 30% (*v*/*v*) hydrogen peroxide and 98% (*v*/*v*) formic acid (in the ratio of 1:9 *v*/*v*) and were subsequently hydrolyzed in the way described above. For tryptophan (Trp) determination, alkaline hydrolysis was performed: about 0.1–0.2 g of freeze-dried sample was hydrolyzed with 10 mL of NaOH 4.2 Mol L^{-1} for 16 h under N_2 flux and neutralized with HCl. The HPLC analyzer (Agilent 1100 Series HPLC, Agilent, Santa Clara, CA, USA) tests were performed by automated online pre-column derivatization using an automated liquid sampler and Poroshell 120 column HPH-C18 (3.0 100 mm, 2.7 lm. P/N 695975–502). For the standard preparation derivatization process, the LC method used was performed according to Agilent Pub. #5990-4547EN (Pub No. 5990-4547EN, 8 October, 2009, Agilent Technologies). The primary amino acids (OPA-derivatized) were monitored at 338 nm. The secondary amino acids (FMOC-derivatized) were monitored at 262 nm. For methionine (Met) and cysteine (Cys) detection, DTDPA (3,3-dithiodipropionic acid) was used as the derivatizing agent instead of FMOC. The separation was carried out under gradient elution with two mobile phases. Phase A: 10 mM NaH_2PO_4 + 10 mM $Na_2B_4O_7$ + 5 mM NaN_3, pH 8.2 adjusted with HCl 5 M, and Phase B: ACN–MeOH–water (45:45:10, *v*/*v*/*v*). The flow rate was 1.00 mL min^{-1}, the column temperature 40 °C, and injection volume 20 µL.

Total lipids were determined using a slightly modified version of the Bligh and Dyer method [26]. An aliquot of lyophilized biomass was mixed with 600 µL of chloroform–methanol (2:1 *v*/*v*), after mixing well, 200 µL of methanol and 333 µL of deionized water were added. The mixtures were then transferred into a separator funnel and shaken for 5 min. The lipid fraction was then collected from the separator funnel and gravimetrically determined after evaporation over one night. For fatty acid compositional analysis, the microalgae lipids were esterified as suggested by the Sigma-Aldrich Fatty Acid Methyl Ester Preparation Protocol and used for GC-MS analysis. Chromatographic analysis was performed using an Agilent 5975C Series GC/MSD and FID as a detector (Agilent, Santa Clara, CA, USA). Volatiles were separated using a polar capillary column Zebron ZBFAME (Zebron, Phenomenex, Castel Maggiore, Italy) of 30 m × 0.25 mm (ID) and a film thickness of 0.20 µm. The injection volume was 1 µL with a split ratio of 20:1. The carrier gas was helium at a flow rate of 1 mL min^{-1}. The temperature program was isothermal for 2 min at 100 °C, then the temperature was raised at a rate of 3 °C min^{-1} to 240 °C and kept at 240 °C for 5 min. Injection temperature was 250 °C, and the transfer line to the mass spectrometer was maintained at 285 °C. The mass spectra were obtained by electronic impact at 70 eV, a multiplier voltage of 1294 V and collecting data at an m/z range of 45–300. Compounds were identified and quantified by comparing their mass spectra and retention times (RT) with those from the standards contained in the Supelco 37 Component

FAME Mix provided by Supelco, Sigma-Aldrich (Saint Louis, MO, USA). Heptadecane was used as an internal standard.

2.7. Data Analysis

Data were processed by one-way ANOVA (analysis of variance), the Tukey's HSD multiple comparison tests ($p < 0.05$) to compare means, and multivariate analyses, i.e., principal component analysis (PCA), using XLSTAT version 2016.02.28451 (New York, NY, USA).

3. Results and Discussion

3.1. Subsection Characterization of Nano-Filtered Permeate (NFP) and Cheese Whey (CW)

In this work, NFP and CW were applied as nutrient and C supplier in substitution of the commercial substrate (BG-11) and CO_2, to grow algae, and, simultaneously, to remove nutrients and C from the wastes employed. In particular, NFP was used to supplement nutrients, as previously studied [12], because of its high nutrient content and very low COD (Table 1). CW was used as a C source to test the ability of the selected algae–microbial consortia for growing under mixotrophic conditions since this substrate had already been tested with success for this purpose [5]. The final culture medium consisted of 80% NFP (v/v), 10% CW (v/v), and 10% algae inoculum (v/v) and presented a TN and C concentration of 169 ± 4 mg N L^{-1} and 1962 ± 63 mg C L^{-1}, respectively. Notably, the final

Table 1. NFP and CW chemical characterization vs. BG-11 nutrient solution.

		NFP	CW	BG-11
pH		8.5 ± 0.0	5.9 ± 0.0	7.4
TN	mg L^{-1}	136 ± 0	601 ± 43	247
NH_4-N	mg L^{-1}	132 ± 2	54 ± 4	19
COD	g L^{-1} O^2	0.1 ± 0.0	62 ± 2	-
P	mg L^{-1}	0.5 ± 0.0	502 ± 45	7.11
Na	mg L^{-1}	250 ± 3	2641 ± 87	414
Mg	mg L^{-1}	5.4 ± 0.1	69 ± 2	7.4
K	mg L^{-1}	188 ± 8	1491 ± 40	17.9
Ca	mg L^{-1}	9.7 ± 0.2	343 ± 12	9.8
Fe	mg L^{-1}	u.d.l [a]	1.8 ± 0.2	1.4
B	mg L^{-1}	0.5 ± 0.1	u.d.l	0.5
Al	mg L^{-1}	0.6 ± 0.0	u.d.l	n.p. [c]
Cr	µg L^{-1}	4.7 ± 0.6	u.d.l	n.p.
Co	µg L^{-1}	4.8 ± 1.2	u.d.l	10
Cu	µg L^{-1}	30.7 ± 0.4	359 ± 94	30
Zn	µg L^{-1}	57 ± 16	212 ± 27	50
Se	µg L^{-1}	5.2 ± 0.4	u.d.l	n.p.
Mo	µg L^{-1}	19 ± 5	70 ± 3	150
Cd	µg L^{-1}	7 [b]	u.d.l	n.p.
Pb	µg L^{-1}	6.5 ± 2.0	u.d.l	n.p.
As	µg L^{-1}	u.d.l	u.d.l	n.p.
Mn	µg L^{-1}	u.d.l	u.d.l	500
Ni	µg L^{-1}	u.d.l	292 ± 25	n.p.

[a] u.d.l refers to under detection level. [b] The other replicates are under detection level. [c] n.p. refers to not present.

P concentration of the culture medium was 50.7 ± 4.5 mg L^{-1} because of the presence of CW, a figure much higher than that of P in both the BG-11 (7.11 mg L^{-1}) and NFP already used to grow these algae consortia under autotrophic conditions [12].

Similar high P dosages were previously reported to have been applied to *Chlorella* under mixotrophic cultivation and no adverse problems were registered [5,27]. CW also contained nitrogen, but it was in organic form and only a small percentage of it was in the form of ammonia (5.4 ± 0.4 mg L^{-1}). CW showed high COD because of the high organic

matter content (Table 1). NFP and CW both contained many other useful nutrients, and the final concentrations were similar to those reported for BG-11 (Table 1).

3.2. Microalgae Consortia Growth

The results (Table 2) indicated that the isolated ACs were capable of growth under mixotrophic conditions, apart from the cultures AC_3 and AC_10 that crashed. It is not a general feature of microalgae to show better growth performance in mixotrophic mode. It has been reported that some mixotrophic strains even failed to gain sufficient autotrophic yield because of the decline in chlorophyll content due to organic carbon supplementation, and downregulation of the Calvin cycle and TCA cycle in mixotrophy [28,29]. Moreover, algae have different heterotrophic potentials in different conditions and so not all algae are capable of growing mixotrophically, even with different carbon sources [30].

Table 2. Algae consortia main genus composition and growing performance.

	Eukaryotic Genus		**Prokaryotic Genus**		**μ (Auto)**	**μ (Mixo)**
	Algae % [a,g]	**Other Eukaryotes % [a,g]**	**Algae % [b,h]**	**Other Prokaryotes % [b,h]**	**d^{-1}**	**d^{-1}**
AC_1	*Chlorella* 99.1%	n.f. [c]	n.f.	*Paludisphaera* 36% (Planctomycetota)	0.55 ± 0.04 a [f]	0.53 ± 0.07 a
AC_2	*Chlorella* 8.4%	*Nuclearia* 40.6%; *Vahlkampfia* 30.7%; *Colpoda* 15.6%	*Synechocystis* 35.9%	*Truepera* 21% (Deinococcata)	0.22 ± 0.03 b	0.47 ± 0.01 a *
AC_3	*Chlorella* 85%	-	*Synechocystis* 19.6%	*SM1A02* 36.8% (Planctomycetota)	0.25 ± 0.04 b	- [e]
AC_4	*Chlorella* 76.4%	*Colpoda* 10.3%	*Synechocystis* 27.9%	*SM1A02* 34.5% (Planctomycetota)	0.31 ± 0.12 b	0.48 ± 0.05 a
AC_5	*Chlorella* 30.6%	*Colpoda* 36.1%; *Nuclearia* 17.7%	*Synechocystis* 84.8%	n.f.	0.29 ± 0.04 b	0.43 ± 0.03 a
AC_6	*Tetradesmus* 85.4%	*Colpoda* 9%	n.f.	Others [d] 61%	0.31 ± 0.02 b	0.43 ± 0.03 a *
AC_7	*Tetradesmus* 42.6%	*Colpoda* 34.8%	*Synechocystis* 21.4%	*Chloronema* (Chloroflexi) 22.9%	0.24 ± 0.02 b	0.57 ± 0.01 a *
AC_8	*Scenedesmus* 8.1%; *Chlorella* 6.3%	*Colpoda* 69.3%	n.f.	*SM1A02* 42.5% (Planctomycetota)	0.28 ± 0.08 b	0.40 ± 0.01 a
AC_9	*Chlorella* 82.3%	*Vermamoeba* 11.9%	*Synechocystis* 35.4%	*SM1A02* 34.3% (Planctomycetota)	0.31 ± 0.02 b	0.61 ± 0.13 a
AC_10	*Tetradesmus* 98.4%	n.f.	*Synechocystis* 54.2%	n.f.	0.52 ± 0.06 a	- [e]
AC_11	*Chlorella* 34.5%	*Cyclidium* 34.1%	*Synechocystis* 9.2%	*SM1A02* 57.7% (Planctomycetota)	0.18 ± 0.01 b	0.51 a
AC_12	*Chlorella* 39.6%; *Tetradesmus* 32.6%	*Vermamoeba* 9.4%	*Synechocystis* 3.6%	*Sandaracinus* 29.8% (Proteobacteria); Others [c] 52.5%	0.58 ± 0.06 a	0.56 ± 0.11 a

[a] Genus composition in microalgae consortium eukaryotic community. [b] Genus composition in microalgae consortium prokaryotic community. [c] Not found. [d] Others refers to undetectable composition in prokaryotic community. [e] Culture crash in AC_3 and AC_10 mixotrophy. [f] Means followed in the same column by the same lower-case letter are not statistically different ($p < 0.05$) according to Tukey test. [g] Percentages refers to the relative abundance of Operational Taxonomic Units (OTUs) assigned to each genus with respect to total OTUs assigned to eukaryotes. [h] Percentages refers to the relative abundance of OTUs assigned to each genus with respect to total OTUs assigned to prokaryotes. '*' Denotes a significant ($p < 0.05$) difference between growth rate values in mixotrophy with respect to autotrophy (Student *t*-test).

It is interesting to compare mixotrophic growth obtained in this work with those obtained for the same algae–microbial consortia cultivated under autotrophic conditions (Table 2) because the literature indicated generally better growth performance in mixotrophy [2,31,32]. For example, it was reported that the mixotrophic growth of *Chlorella* or *Tetradesmus* was effective enough to increase the growth rate and biomass productivity [33–35].

High increases (about double) of the growing kinetic (Table 2) for mixotrophy were found for AC_2, AC_6, and AC_7 (student t-test), while for the rest of the cultures, namely AC_1, AC_4, AC_5, AC_8, AC_9, and AC-12, the increase was minor. On average, the specific growth rates (μ) of the ACs in mixotrophic cultivation (this work) was higher than those of autotrophic cultivation [12] (+47%), i.e., 0.34 ± 0.14 d^{-1} (n = 12) and 0.50 ± 0.04 d^{-1} (n = 10), respectively. It was remarkable that the fastest-growing ACs in autotrophy, i.e., AC_1, and AC_12 (Table 1) [12], showed no significant difference in mixotrophy and that the specific growth rates registered were similar to those obtained for the other ACs under mixotrophic conditions (Table 2). Moreover, data reported (specific growth rates) (Table 2), indicated that under mixotrophic conditions, the variability in AC growth was reduced considerably in comparison with that under autotrophic conditions previously studied [12]. It is probable that some factors limited AC growth under autotrophic conditions, i.e., C availability, which can be overcome with the addition of C (as CW) (this work). The fact that AC_1 and AC_12 did not increase their growth performance under mixotrophy can be explained by the fact that they had reached maximum growth ability under batch conditions. Mixotrophic growth of microalgae requires light to fix CO_2 by photosynthesis as well as to take up organic carbon: its provision in PBRs must be adequate in terms of intensity and duration [36]. The light was probably attenuated in a PBR due to the significant mutual shading effects that occurred among microalgal cells, especially for the two fastest-growing cultures (AC_1 and AC_12). This fact showed some limits in the culture method chosen to grow algae, although this work mainly aimed to test the ability of these newly isolated algae–microbial consortia to grow under mixotrophic conditions (see Introduction section).

In any case, the growth rates found for the ACs were in line with those reported for *Chlorella* sp. in mixotrophic conditions performed with similar organic carbon concentrations to those adopted in this work, i.e., 0.49 ± 0.25 d^{-1} (n = 5) [2,27,35,37,38].

3.3. Nutrient Mass Balance

Microalgae in different cultivation modes would affect the algae's intracellular metabolism, resulting in different behaviours in nutrient removal. The prominent feature of mixotrophic culture is the presence of two energy sources, organic carbon sources and light, so that microalgae can benefit from both photoautotrophic and heterotrophic growth [36]. Thus, this work aimed not only to test the growth ability of ACs in mixotrophic cultivation but also to evaluate their ability to remove nutrients, compared to the performance in autotrophic mode.

Results obtained (Table 3) indicated a high nitrogen uptake by biomass, i.e., 82.5 ± 9.5% $TN_{initial}$ (n = 10), on average. This value is 35.2% higher than that obtained under autotrophic conditions i.e., 61 ± 15% $TN_{initial}$ (n = 12), in a previous study [12]. This agrees with a finding that mixotrophic conditions stimulate algae consortia growth, which allowed a higher N uptake [39]. Data obtained agreed, also, with literature data reported for both *Chlorella* sp. and *Tetradesmus* sp. cultivated in mixotrophic mode, i.e., 90.8 ± 10.4% $TN_{initial}$ (n = 7) [40,41] and 87.3 ± 14.6% $TN_{initial}$ (n = 16) [42–45].

Phosphorus is assimilated by algae as essential inorganic orthophosphate to produce phospholipids, ATP, and nucleic acids [46] as well as high-value-added products such as astaxanthin and polyunsaturated fatty acids [47]. Phosphorus can be removed from media through both biological assimilation and chemical precipitation. In this study, since the pH measured for culture under a mixotrophic condition was not high enough to trigger precipitation, P uptake was probably the only mechanism explaining P removal [23]. P uptake, measured for all ACs, was considered high, i.e., 91.8 ± 7.5% $P_{initial}$ (n = 10) on average. This showed the high efficiency of ACs in removing P under mixotrophic conditions [41,48]. This ability was similar to those previously reported for both *Chlorella* sp. and *Tetradesmus* sp. cultivated mixotrophically, i.e., 87.8 ± 11.4% $P_{initial}$ (n = 8) [40,41] and 86.7 ± 7.3% $P_{initial}$ (n = 14) [42,43,45]. These data are of interest, taking into consideration that P applied under mixotrophy was in excess in relation to the amount required for algae

to grow, i.e., $P_{initial}$ of 51 ± 5 mg L^{-1}. This rate was much higher than that dosed previously in autotrophy, i.e., $P_{initial}$ of 7.61 mg L^{-1} [12]. The literature reported that microalgae are capable of taking up and storing more P than they need whenever it becomes available [49].

Table 3. Nitrogen, phosphorus and carbon mass balance.

AC	$TN_{initial}$ [a] mg L^{-1}	TN_{final} [b] mg L^{-1}	$N_{biomass}$ [c] g kg^{-1} DM [i]	N Uptaken by Biomass % $TN_{initial}$ [a]	$P_{initial}$ [d] mg L^{-1}	P_{final} [e] mg L^{-1}	P Uptaken by Biomass % $P_{initial}$ [d]	$C_{initial}$ [f] mg L^{-1}	C_{final} [g] mg L^{-1}	TOC Removal % $TOC_{initial}$
AC_1	169 ± 4	17 ± 2	61 ± 0	87 ± 2	51 ± 5	2 ± 1	96 ± 2	1962 ± 63	171 ± 21	91 ± 2
AC_2	169 ± 4	30 ± 5	69 ± 1	87 ± 1	51 ± 5	1 ± 0	98 ± 1	1962 ± 63	261 ± 39	87 ± 3
AC_3					- [h]					
AC_4	169 ± 4	10 ± 1	44 ± 0	87 ± 1	51 ± 5	1 ± 0	99 ± 0	1962 ± 63	267 ± 31	86 ± 2
AC_5	169 ± 4	14 ± 2	47 ± 0	79 ± 1	51 ± 5	1 ± 0	97 ± 1	1962 ± 63	295 ± 17	85 ± 1
AC_6	169 ± 4	22 ± 1	76 ± 1	84 ± 3	51 ± 5	5 ± 0	91 ± 1	1962 ± 63	233 ± 48	88 ± 3
AC_7	169 ± 4	22 ± 10	48 ± 2	95 ± 4	51 ± 5	9± 1	81 ± 1	1962 ± 63	242 ± 4	88 ± 0
AC_8	169 ± 4	11 ± 1	63 ± 1	94 ± 3	51 ± 5	2 ± 0	97 ± 0	1962 ± 63	139 ± 19	93 ± 1
AC_9	169 ± 4	33 ± 0	70 ± 2	67 ± 1	51 ± 5	2 ± 0	96 ± 1	1962 ± 63	260 ± 36	89 ± 3
AC_10					- [h]					
AC_11 [j]	169 ± 4	39	85	71	51 ± 5	9	82	1962 ± 63	175	91
AC_12	169 ± 4	51 ± 19	65 ± 1	74 ± 2	51 ± 5	9 ± 1	81 ± 1	1962 ± 63	395 ± 24	80 ± 17

[a] Initial TN concentration of culture medium at the start of the experiments. [b] TN concentration of culture medium at the end of the experiments. [c] TN concentration in AC biomass. [d] Initial P concentration of culture medium at the start of the experiments. [e] P concentration of culture medium at the end of the experiments. [f] Initial C concentration of culture medium at the start of the experiments. [g] concentration of culture medium at the end of the experiments. [h] Culture crashed. [i] DM refers to dry matter. [j] The replicate culture of AC_11 failed.

TOC reduction was 88 ± 4% $TOC_{initial}$ (n = 10), on average. This was comparable to 86% carbon removal obtained with *Chlorella vulgaris* [50] and lower than the 98% carbon removal obtained with mixed algal culture [51]. Again, these data were much higher than those reported for *Tetradesmus* sp., i.e., 66 ± 8% $TOC_{initial}$ (n = 2) grown mixotrophically [46].

According to [52], simultaneous inorganic and organic carbon assimilation may offer an opportunity to effectively cultivate microalgae and improve nutrient removal. Therefore, it can be deduced that the organic matter in the waste streams promoted the mixotrophic growth of algae consortia, as well as their performance in removing nitrogen, phosphorus, and carbon.

3.4. Biochemical Composition of AC Biomasses

Proteins, lipids and carbohydrates are the main microalgal cell components, which are estimated to be about 60–85% of microalgal dry weight [53]. Studies demonstrated that changes in the cellular biochemical composition were influenced by the trophic conditions, implying that mixotrophic conditions changed the metabolic pathways of nitrogen and carbon [38]. Therefore, the biochemical composition of ACs was studied and compared to algae–microbial consortia cultivated in the autotrophic mode (Table 4).

The average protein content in mixotrophy was, on an average basis, 393 ± 83 g kg^{-1} DM (n = 10). This contents was comparable to that reported in the literature for *Chlorella* sp. cultivated mixotrophically, i.e., 425 ± 134 g kg^{-1} DM (n = 5) [2,7] and higher than that reported for *Tetradesmus* sp. cultivated mixotrophically, i.e., 215 ± 17 g kg^{-1} DM (n = 4) [54,55]. Significant increases in protein content (AC_1, AC_5, AC_6, AC_9, and AC_11) ($p < 0.05$) were found in mixotrophy compared to autotrophy (Table 4). AC_11 gained the most abundant protein content, accounting for 534 ± 5 g kg^{-1} DM, which was 56.7% higher than that obtained under autotrophy. It seems that microalgal protein production was considerably enhanced by supplementing carbon sources. Similar results have also been previously reported: Melo et al. [56] studied the growth *of Chlorella vulgaris* by supplementing three kinds of agro-industrial by-products as organic sources. The highest protein content was obtained by adding 1% corn steep liquor, and the protein content was about twice as high as that in autotrophic conditions. Kadkhodaei et al. [57],

provided data for *Dunaliella salina* showing four times more protein production than a control by adding glucose to the medium, suggesting that this metabolism is promising for protein production.

Table 4. Biochemical composition comparisons between autotrophy and mixotrophy.

AC	Autotrophy			Mixotrophy		
	Proteins g kg^{-1} DM	**Lipids g kg^{-1} DM**	**Carbohydrates g kg^{-1} DM**	**Proteins g kg^{-1} DM**	**Lipids g kg^{-1} DM**	**Carbohydrates g kg^{-1} DM**
AC_1	257 ± 0 g [b]	119 ± 1 fg	596 ± 4 a	381 ± 1 e *	121 ± 7 bcd	470 ± 7 cde
AC_2	460 ± 4 b	105 ± 7 g	405 ± 9 ef	430 ± 4 c	75 ± 6 e *	469 ± 8 de
AC_3	561 ± 1 a	152 ± 10 cde	254 ± 11 g		- [a]	
AC_4	422 ± 3 c	177 ± 3 b	359 ± 6 f	276 ± 1 g *	118 ± 5 cd *	573 ± 5 a
AC_5	266 ± 0 g	153 ± 9 bcde	565 ± 9 ab	295 ± 1 fg *	134 ± 1 b	536 ± 2 ab
AC_6	305 ± 5 f	173 ± 11 bc	486 ± 12 cd	474 ± 1 b *	106 ± 7 d *	392 ± 7 f
AC_7	334 ± 4 e	128 ± 8 efg	512 ± 9 bc	301 ± 14 f	151 ± 7 a *	527 ± 16 abc
AC_8	420 ± 2 c	177 ± 9 b	369 ± 10 f	394 ± 8 de	124 ± 6 bc *	447 ± 11 e
AC_9	398 ± 2 cd	178 ± 8 b	406 ± 8 ef	436 ± 3 c *	68 ± 5 e *	460 ± 6 de
AC_10	382 ± 7 d	156 ± 9 bcd	425 ± 9 def		- [a]	
AC_11	341 ± 2 e	135 ± 4 def	494 ± 5 c	534 ± 5 a *	77 ± 3 e *	348 ± 6 f
AC_12	273 ± 22 g	230 ± 15 a	472 ± 27 cde	405 ± 2 d	63 ± 4 e *	501 ± 4 bcd

[a] Culture crashed. [b] Means followed in the same column by the same lower-case letter are not statistically different ($p < 0.05$) according to Tukey test. '*' Denotes a significant ($p < 0.05$) difference between protein or lipid values in mixotrophy with respect to autotrophy (Student *t*-test).

However, for AC_2, AC_7, AC_8, and AC_12 consortia, there were no significant differences in protein content ($p < 0.05$) between the two cultivation modalities. This result was in accordance with those reported for *Chlorella* sp. growing mixotrophically on three agro-food by-products, i.e., cheese whey, white wine lees, and glycerol [5], and *Chlorella vulgaris* growing on industrial dairy waste [2].

The lipid contents of ACs cultivated in the mixotrophic condition were, on average, 103.6 ± 30.6 g kg^{-1}DM ($n = 10$), which was lower than the averages reported for *Chlorella* sp. cultivated mixotrophically, i.e., 172 ± 98 g kg^{-1} DM ($n = 21$) [7,58,59] and *Tetradesmus* sp. grown mixotrophically, i.e., 221 ± 79 g kg^{-1} DM ($n = 22$) [40,43,45,54,60].

The lipid contents showed the opposite trend in comparison with the protein contents of the two cultivation modes. In particular, except for AC_7, either a significant decrease (AC_2, AC_4, AC_6, AC_8, AC_9, AC_11, and AC_12) or no significant differences (AC_1 and AC_5) of lipid contents in mixotrophy were found ($p < 0.05$) compared to autotrophy. These results agreed with those reported for *Chlorella* sp. by Liang et al. [7] who found a decrease in the lipid contents in mixotrophic cultures and those reported by Salati et al. [5] who found no significant changes in the lipid contents in mixotrophic cultures compared to the autotrophic ones.

The carbohydrate content in ACs presented an average content of 472 ± 67 g kg^{-1} DM ($n = 10$): this value was similar to those of ACs cultivated in autotrophic conditions, i.e., 445 ± 95 g kg^{-1} DM ($n = 12$). Compared with pure algae cultures, the carbohydrate content in ACs was found to be higher than in *Chlorella* sp. and *Tetradesmus* sp. grown mixotrophically i.e., 361 ± 111 g kg^{-1}DM ($n = 5$) [7,48] and 224 ± 39 g kg^{-1}DM ($n = 16$) [45,54,55], respectively. High carbohydrate contents in AC culture have been noted because of the bacterial community. It has been reported that carbohydrate production can be significantly enhanced by the co-culture of algae with bacteria in both autotrophic and mixotrophic conditions [61].

3.5. Amino Acids (AA) and Fatty Acids (FA) Speciation

3.5.1. Amino Acids (AA)

Proteins are composed of different amino acids, and thus, the quality of proteins can vary depending on the availability of essential amino acids. Plant proteins are often

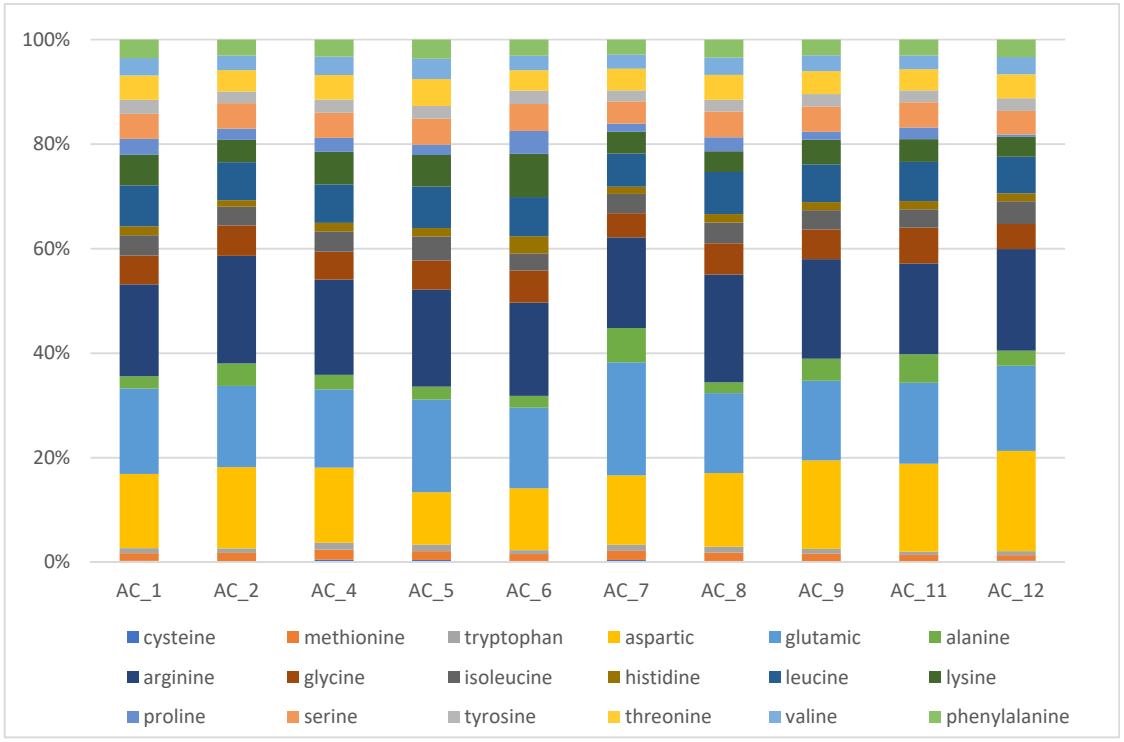
considered an incomplete protein source as they commonly lack one or more of the essential amino acids. Algae are generally regarded as a viable protein source, with essential amino acid (EAA) composition meeting FAO requirements, and they are on par with other protein sources, such as soybean and egg [62].

In this work, eighteen amino acids (AAs) and the complete profile of all nine essential amino acids (EAAs) were detected in ACs grown in mixotrophic conditions (Figure 1a and Table S2). Data indicated that mixotrophic conditions did not affect amino acid composition in comparison with autotrophy [12] and that the dominating non-essential amino acids (NEAAs) and essential amino acids (EAAs) were similar as well (Figure 1a). In particular, the dominating AAs in all cultures were arginine (Arg), glutamic acid (Glu), and aspartic acid (Asp), followed by leucine (Leu), glycine (Gly), lysine (Lys), serine (Ser), threonine (Thr), and phenylalanine (Phe). The most abundant NEAAs in all ACs reported as average values (g 100 g^{-1} crude protein) (n = 10) were: Arg (18.7 ± 1.2), Glu (16.4 ± 1.9), and Asp (14.6 ± 2.5), and the most abundant EAAs were: Leu (7.4 ± 0.5), Lys (5.2 ± 1.4), and Thr (4.4 ± 0.4). These data were mostly in line with data reported for *Chlorella* sp. grown mixotrophically (data reported as g 100 g^{-1} crude protein) (n = 7) [5,63], i.e., Arg (7.3 ± 2.3), Glu (11 ± 1), and Asp (10.1 ± 9.7) as NEAAs and Leu (9.6 ± 0.7), Lys (6.3 ± 1.4), and Thr (4 ± 1) as EAAs. Higher Glu and Arg content (NEAAs) was found compared to that reported in the above literature because the $N_{initial}$ source in this work was mainly represented by ammonium that is quickly assimilated by algal cells, avoiding toxicity, leading to a net synthesis of Glu [64]. Arg, as well, has been reported to be preferentially produced by algae when ammonium is the N source [65].

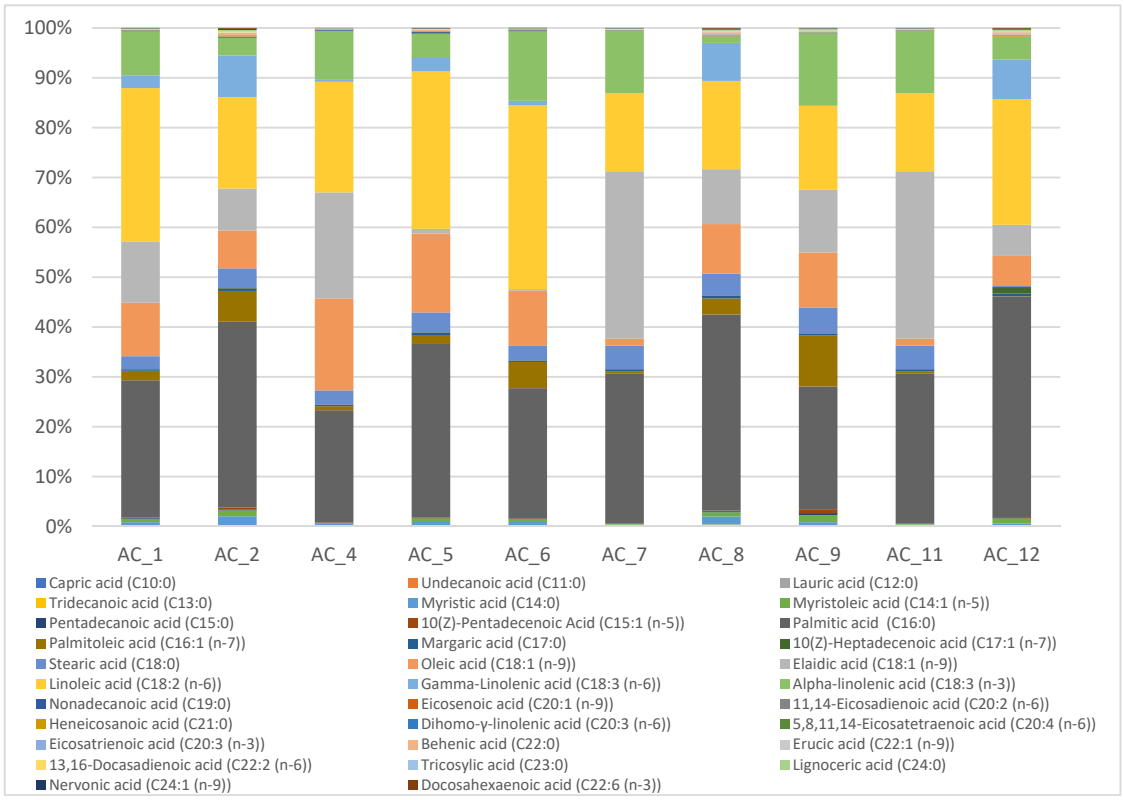
The EAA content of algae consortia studied in this work was, on average, 31.4 ± 2.7 g 100 g^{-1} crude protein; lower than that reported for pure *Chlorella* sp. grown mixotrophically, i.e., 42.3 ± 1.4 g 100 g^{-1} crude protein (n = 7) [5,63]. It is worth noting that EAA content was higher under mixotrophic conditions than under autotrophy, i.e., 24.3 ± 2.8 g 100 g^{-1} crude protein [12]. This result indicated that mixotrophic cultivation of algae consortia did not much affect the amino acid composition pattern compared to that obtained in autotrophy but increased the essential amino acid contents.

Apart from EAA contents, the amino acid score (AAS) of the first limiting amino acid is also a key factor in assessing protein quality: the abundance of the first limiting amino acid determines the utilization efficiency of all essential amino acids for protein synthesis [66]. A higher AAS value should yield a higher protein utilization rate or bio-efficiency in the body. Table S1 shows the AAS value of the ten ACs, calculated as proposed by FAO standards [67]. AAS ranged from 0.6 to 1, where the limiting amino acids were Val and SAA (Met + Cys) for all ACs; these figures are quite similar to data reported in the literature. It was reported that *Chlorella vulgaris* and *Chlorella sorokiniana* had AAS values of 1.10 and 1.16, respectively, while *Acutodesmus obliquus* had an AAS value of 0.86 [66]. Kent et al. [68] reported that the microalgae *Nannochloropsis* sp., *Scenedesmus* sp., *Dunaliella* sp., and *Chlorophyta* sp. had AAS values of 0.98–1.05, and two microalgae products derived from *Spirulina* and *Chlorella* had AAS values of 0.81 and 0.92, respectively.

AA are molecules that combine to form proteins, and thus the AA composition is critical in establishing the nutritional value of algae–microbial consortia. Figure 2a is the principal component analysis of the distribution of each AA in ten ACs. Two main factors (PCs), accounting for 64.23% of the total variance, i.e., PC1 32.53% and PC2 31.70% of the total variance, were determined. Three groups can be assigned based on EAA locations in Figure 2a since EAA, located in the top right part, indicated that EAA content was positively related to AC_1, AC_4, AC_5, AC_6, and AC_8 (group 1) with an average content of 34 ± 1 g 100 g^{-1} crude protein (n = 5), while for the rest of the ACs, this value was 29 ± 1 g 100 g^{-1} crude protein (n = 5).



(**a**)



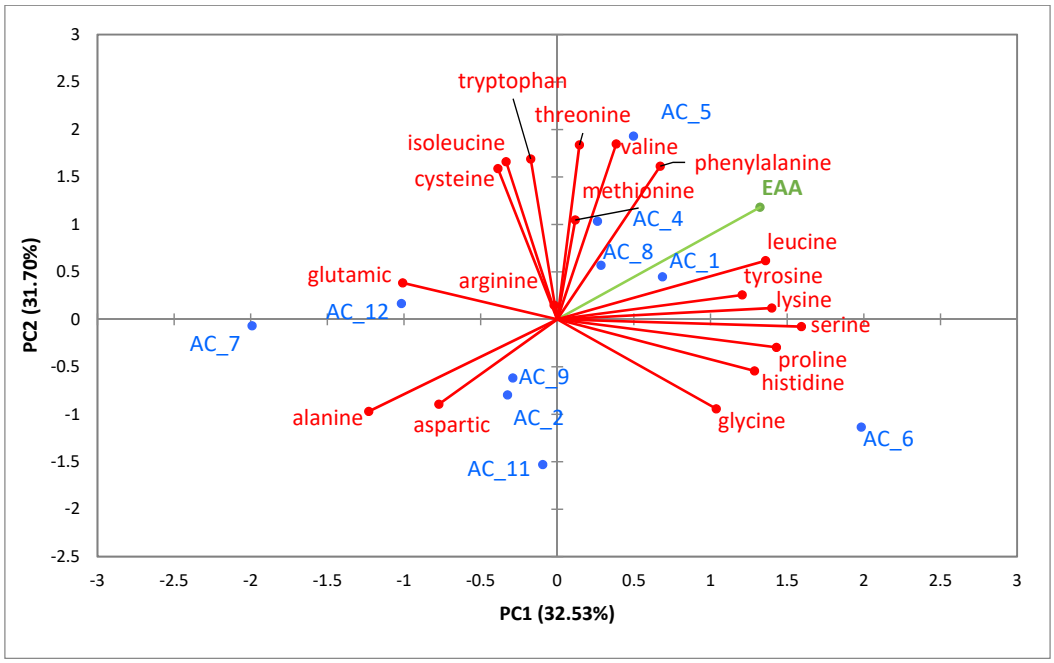
(**b**)

Figure 1. *Cont.*

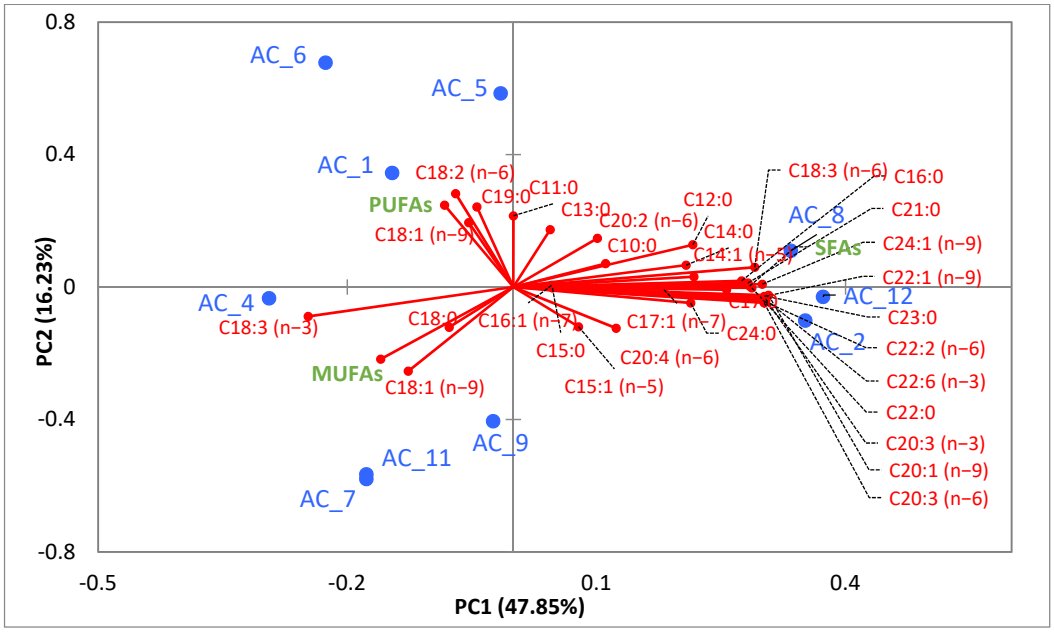
		AC_1	AC_2	AC_4	AC_5	AC_6	AC_7	AC_8	AC_9	AC_11	AC_12
SFAs	% total lipids	31.8	45	26.6	41	30.7	35.6	47.2	32	35.6	46.5
MUFAs	% total lipids	25.9	24.3	40.8	19.6	17.4	36	25.6	36.3	36	15.3
PUFAs	% total lipids	42.3	30.5	32.5	39.3	51.9	28.3	27	31.6	28.3	38
ω6–ω3	/	3.8	7.2	2.3	6.9	2.7	1.3	16.9	1.2	1.3	7.1

(**c**)

Figure 1. (**a**) AA speciation (a); (**b**) FA speciation; (**c**) the summary of FA compositions.



(**a**)



(**b**)

Figure 2. (**a**) Principal component plots for AC vs. amino acids; (**b**) principal component plots for AC vs. fatty acids.

In conclusion, taking into consideration both EAA content and AAS, AC_5 and AC_4 showed the best protein quality regarding their potential as a feed supplement.

3.5.2. Fatty Acids (FA)

To understand if changing the cultivation mode from autotrophy to mixotrophy affects the FA composition, FAs were studied. As a result, thirty-two fatty acids were identified in ten AC cultures. The FAs' distribution in each AC was very similar to the pattern obtained for the autotrophic condition [12], indicating that changing cultivation mode did not affect the FA profile, as previously reported by Li et al. [69].

More precisely, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) detected in this study were 37.2 ± 6.9%, 27.7 ± 8.5%, and 35 ± 7% of the total lipid content (n = 10) (Figure 1b,c), respectively. These figures were in line with data reported for *Chlorella* sp. grown mixotrophically with different carbon sources, i.e., SFAs of 35.5 ± 7.1%, MUFAs of 36.8 ± 9.5%, and PUFAs of 25.6 ± 7.3% of total lipids (n = 5), respectively [37,70–73]. The dominating FAs among all cultures were palmitic acid (C16:0) (31.6 ± 7.0% of total lipids), followed by stearic acid (C18:0) (3.6 ± 1.4% of total lipids). Linoleic acid (C18:2) (23.1 ± 7.3% of total lipids) was dominant among the unsaturated fatty acids, followed by elaidic acid (C18:1 trans-9) (14 ± 11% of total lipids), which was higher than oleic acid (C18:1 cis-9) (9.3 ± 5.2% of total lipids) and equal to alpha-linolenic acid (C18:3) (8.6 ± 4.5% of total lipids), on average (n = 10) (Figure 1b). These results were in line, also, with data reported for mixotrophic algae consortia grown on municipal wastewater [74], where the dominant saturated FA was C16:0 (42.3% of total lipids) and the dominant unsaturated FA was C18:1 (10.9% of total lipids).

To better understand FA distribution across AC, PCA was performed (Figure 2b). Two main PCs explaining 64.08% of the total variance, i.e., PC1 47.85% and PC2 16.23%, were obtained. Since PC1 contained a higher variance value, from Figure 2b, it can be concluded that higher SFAs led to lower PUFA and MUFA contents in ACs. In particular, three distinct groups were detected: (i) AC_1, AC_4, AC_5, and AC_6 that were positively related to PUFAs, (ii) AC_7, AC_9, and AC_11 that were positively related to MUFAs, and (iii) AC_2, AC_8, and AC_12 that were positively related to SFAs.

Among various fatty acids, omega-6 (ω-6) and omega-3 (ω-3) fatty acids are proving indispensable in a properly maintained ratio for numerous biological, physiological, and beneficial health functions [75]. From a nutritional aspect, the ω-6–ω-3 ratio is significant for human/animal health, where the optimum range for diet should be from 1:1 to 4:1 [76].

From Figure 1c, it can be seen that AC_1, AC_4, AC_6, AC_7, AC_9, and AC_11 showed a range below 4:1. Under consideration of the PUFA contents, AC_6 and AC_1 illustrate the greatest interest as feed ingredients due to the abundant amount of PUFA, and as a source of omega-3 supplements to improve human/animal diets by lowering the ω-6–ω-3 ratio.

4. Conclusions

Ten out of twelve microalgae–microbial consortia survived in mixotrophic conditions, indicating that not all ACs can adapt well to the mixotrophic growing mode. However, the well-grown ones obtained 47% higher growth rates in mixotrophic cultivation compared to those in autotrophic cultivation with a remarkable nitrogen uptake ability, i.e., 82.5 ± 9.5% $TN_{initial}$ (n = 10) (35.2% higher than that in autotrophy), 91.8 ± 7.5% $P_{initial}$ (n = 10), and 88 ± 4% $TOC_{initial}$ (n = 10), on an average basis. AC_8 exhibited the best nutrient removal ability in mixotrophic mode, namely, 94 ± 3% $TN_{initial}$, 97 ± 0% $P_{initial}$, and 93 ± 1% $TOC_{initial}$. AA and FA composition were not affected significantly in different cultivation modes, but mixotrophy increased EAA content. AC_5 and AC_4 showed the best protein quality based on EAA content and AAS value, suggesting the potential use of these consortia for producing high quality protein as feed supplements. On the other hand, AC_6 and AC_1, because of low ω-6–ω-3, were suitable for producing biomass to act as omega-3 supplements. Mixotrophic cultivation of ACs using CW can be considered a feasible strat-

egy to reduce costs by increasing the microalgal biomass specific growth rates, while also contributing to the recovery of nutrients from CW and NFP for waste valorization.

This study provides essential guidance for large scale cultivation using indigenous and autochthonous algae–microbial consortia grown on wastewaters in similar conditions. This kind of selection could also be a process upgrade for microalgae-based wastewater treatment plants since, at present, these rely on spontaneous algae blooms [77].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8100474/s1, Table S1: Amino acid score of ACs, Table S2: Amino acids of ACs.

Author Contributions: Conceptualization, M.S., M.D. and F.A.; methodology, M.D. and G.D.; software, M.S.; validation, M.D. and G.D.; formal analysis, M.S., M.D. and B.S.; investigation, M.S. and M.D.; resources, G.D. and F.A.; writing—original draft preparation, M.S.; writing—review and editing, F.A. and M.D.; visualization, M.S.; supervision, F.A.; project administration, F.A.; funding acquisition, F.A. All authors have read and agreed to the published version of the manuscript.

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References

1. SundarRajan, P.S.; Gopinath, K.P.; Greetham, D.; Antonysamy, A.J. A review on cleaner production of biofuel feedstock from integrated CO_2 sequestration and wastewater treatment system. *J. Clean. Prod.* **2019**, *210*, 445–458. [CrossRef]
2. Abreu, A.P.; Fernandes, B.; Vicente, A.A.; Teixeira, J.; Dragone, G. Mixotrophic cultivation of *Chlorella vulgaris* using industrial dairy waste as organic carbon source. *Bioresour. Technol.* **2012**, *118*, 61–66. [CrossRef] [PubMed]
3. Li, T.; Zheng, Y.; Yu, L.; Chen, S. Mixotrophic cultivation of a *Chlorella sorokiniana* strain for enhanced biomass and lipid production. *Biomass Bioenergy* **2014**, *66*, 204–213. [CrossRef]
4. Cecchin, M.; Benfatto, S.; Griggio, F.; Mori, A.; Cazzaniga, S.; Vitulo, N.; Delledonne, M.; Ballottari, M. Molecular basis of autotrophic vs mixotrophic growth in *Chlorella sorokiniana*. *Sci. Rep.* **2018**, *8*, 6465. [CrossRef]
5. Salati, S.; D'Imporzano, G.; Menin, B.; Veronesi, D.; Scaglia, B.; Abbruscato, P.; Mariani, P.; Adani, F. Mixotrophic cultivation of *Chlorella* for local protein production using agro-food by-products. *Bioresour. Technol.* **2017**, *230*, 82–89. [CrossRef]
6. Bhatnagar, A.; Chinnasamy, S.; Singh, M.; Das, K.C. Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters. *Appl. Energy* **2011**, *88*, 3425–3431. [CrossRef]
7. Liang, Y.; Sarkany, N.; Cui, Y. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnol. Lett.* **2009**, *31*, 1043–1049. [CrossRef]
8. Italy: Whey Powder from Cheese (Regions). Available online: https://www.clal.it/en/index.php?section=siero_regioni (accessed on 19 July 2022).
9. Acién, F.G.; Fernández, J.M.; Magán, J.J.; Molina, E. Production cost of a real microalgae production plant and strategies to reduce it. *Biotechnol. Adv.* **2012**, *30*, 1344–1353. [CrossRef]
10. Vo Hoang Nhat, P.; Ngo, H.H.; Guo, W.S.; Chang, S.W.; Nguyen, D.D.; Nguyen, P.D.; Bui, X.T.; Zhang, X.B.; Guo, J.B. Can algae-based technologies be an affordable green process for biofuel production and wastewater remediation? *Bioresour. Technol.* **2018**, *256*, 491–501. [CrossRef]
11. Acién Fernández, F.G.; Gómez-Serrano, C.; Fernández-Sevilla, J.M. Recovery of nutrients from wastewaters using microalgae. *Front. Sustain. Food Syst.* **2018**, *2*, 59. [CrossRef]
12. Su, M.; Dell'orto, M.; Scaglia, B.; D'imporzano, G.; Bani, A.; Adani, F. Growth performance, biochemical composition and nutrient recovery ability of twelve microalgae consortia isolated from various local organic wastes grown on nano-filtered pig slurry. *Molecules* **2022**, *27*, 422. [CrossRef]

13. Devi, N.D.; Sun, X.; Ding, L.; Goud, V.V.; Hu, B. Mixotrophic growth regime of novel strain *Scenedesmus* sp. DDVG I in municipal wastewater for concomitant bioremediation and valorization of biomass. *J. Clean. Prod.* **2022**, *365*, 132834. [CrossRef]
14. Ahmad, A.; Banat, F.; Alsafar, H.; Hasan, S.W. Algae biotechnology for industrial wastewater treatment, bioenergy production, and high-value bioproducts. *Sci. Total Environ.* **2022**, *806*, 150585. [CrossRef]
15. Ferreira, A.; Molnar Jazić, J.; Gouveia, L.; Maletić, S.; Tomić, M.; Agbaba, J.; Vladić, J. Valorisation of microalga *Tetradesmus obliquus* grown in brewery wastewater using subcritical water extraction towards zero waste. *Chem. Eng. J.* **2022**, *437*, 135324. [CrossRef]
16. Wang, S.; Yin, C.; Yang, Z.; Hu, X.; Liu, Z.; Song, W. Assessing the potential of *Chlorella* sp. for treatment and resource utilization of brewery wastewater coupled with bioproduct production. *J. Clean. Prod.* **2022**, *367*, 132939. [CrossRef]
17. Su, M.; Dell'Orto, M.; D'Imporzano, G.; Bani, A.; Dumbrell, A.J.; Adani, F. The structure and diversity of microalgae-microbial consortia isolated from various local organic wastes. *Bioresour. Technol.* **2022**, *347*, 126416. [CrossRef] [PubMed]
18. Subashchandrabose, S.R.; Ramakrishnan, B.; Megharaj, M.; Venkateswarlu, K.; Naidu, R. Consortia of cyanobacteria/microalgae and bacteria: Biotechnological potential. *Biotechnol. Adv.* **2011**, *29*, 896–907. [CrossRef] [PubMed]
19. APHA (American Public Health Association). *Standard Methods for the Examination of Water and Wastewater*; APHA: Washington, DC, USA, 2005.
20. Travieso, L.; Benítez, F.; Sánchez, E.; Borja, R.; Martín, A.; Colmenarejo, M.F. Batch mixed culture of *Chlorella vulgaris* using settled and diluted piggery waste. *Ecol. Eng.* **2006**, *28*, 158–165. [CrossRef]
21. Sforza, E.; Cipriani, R.; Morosinotto, T.; Bertucco, A.; Giacometti, G.M. Excess CO_2 supply inhibits mixotrophic growth of *Chlorella protothecoides* and *Nannochloropsis salina*. *Bioresour. Technol.* **2012**, *104*, 523–529. [CrossRef]
22. Cerón García, M.C.; Sánchez Mirón, A.; Fernández Sevilla, J.M.; Molina Grima, E.; García Camacho, F. Mixotrophic growth of the microalga phaeodactylum tricornutum: Influence of different nitrogen and organic carbon sources on productivity and biomass composition. *Process Biochem.* **2005**, *40*, 297–305. [CrossRef]
23. Silva-Benavides, A.M.; Torzillo, G. Nitrogen and phosphorus removal through laboratory batch cultures of microalga *Chlorella vulgaris* and cyanobacterium *Planktothrix isothrix* grown as monoalgal and as co-cultures. *J. Appl. Phycol.* **2012**, *24*, 267–276. [CrossRef]
24. Mišurcová, L.; Buňka, F.; Vávra Ambrožová, J.; Machů, L.; Samek, D.; Kráčmar, S. Amino acid composition of algal products and its contribution to RDI. *Food Chem.* **2014**, *151*, 120–125. [CrossRef] [PubMed]
25. Wilkie, A.C.; Mulbry, W.W. Recovery of dairy manure nutrients by benthic freshwater algae. *Elsevier* **2002**, *84*, 81–91. [CrossRef]
26. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Philos.* **1990**, *20*, 215–240.
27. Kim, S.; eun Park, J.; Cho, Y.B.; Hwang, S.J. Growth rate, organic carbon and nutrient removal rates of *Chlorella sorokiniana* in autotrophic, heterotrophic and mixotrophic conditions. *Bioresour. Technol.* **2013**, *144*, 8–13. [CrossRef]
28. Patel, A.K.; Joun, J.M.; Hong, M.E.; Sim, S.J. Effect of light conditions on mixotrophic cultivation of green microalgae. *Bioresour. Technol.* **2019**, *282*, 245–253. [CrossRef]
29. Subramanian, G.; Yadav, G.; Sen, R. Rationally Leveraging mixotrophic growth of microalgae in different photobioreactor configurations for reducing the carbon footprint of an algal biorefinery: A techno-economic perspective. *RSC Adv.* **2016**, *6*, 72897–72904. [CrossRef]
30. Nicodemou, A.; Kallis, M.; Agapiou, A.; Markidou, A.; Koutinas, M. The effect of trophic modes on biomass and lipid production of five microalgal strains. *Water* **2022**, *14*, 240. [CrossRef]
31. Prasad, R.; Gupta, S.K.; Shabnam, N.; Oliveira, C.Y.B.; Nema, A.K.; Ansari, F.A.; Bux, F. Role of microalgae in global CO_2 sequestration : Physiological mechanism, recent development, challenges, and future prospective. *Sustainability* **2021**, *13*, 23. [CrossRef]
32. Park, K.C.; Whitney, C.; Mcnichol, J.C.; Dickinson, K.E.; Macquarrie, S.; Skrupski, B.P.; Zou, J.; Wilson, K.E.; Leary, S.J.B.O.; Mcginn, P.J. Mixotrophic and photoautotrophic cultivation of 14 microalgae isolates from Saskatchewan, Canada : Potential applications for wastewater remediation for biofuel production. *J. Appl. Phycol.* **2012**, *24*, 339–348. [CrossRef]
33. Combres, C.; Laliberté, G.; Reyssac, J.S.; de la Noüe, J. Effect of acetate on growth and ammonium uptake in the microalga *Scenedesmus obliquus*. *Physiol. Plant.* **1994**, *91*, 729–734. [CrossRef]
34. D'Imporzano, G.; Veronesi, D.; Salati, S.; Adani, F. Carbon and nutrient recovery in the cultivation of *Chlorella vulgaris*: A life cycle assessment approach to comparing environmental performance. *J. Clean. Prod.* **2018**, *194*, 685–694. [CrossRef]
35. Wan, M.; Liu, P.; Xia, J.; Rosenberg, J.N.; Oyler, G.A.; Betenbaugh, M.J.; Nie, Z.; Qiu, G. The Effect of mixotrophy on microalgal growth, lipid content, and expression levels of three pathway genes in *Chlorella sorokiniana*. *Appl. Microbiol. Biotechnol.* **2011**, *91*, 835–844. [CrossRef]
36. Carvalho, A.P.; Silva, S.O.; Baptista, J.M.; Malcata, F.X. Light requirements in microalgal photobioreactors : An overview of biophotonic aspects. *Appl. Microbiol. Biotechnol.* **2011**, *89*, 1275–1288. [CrossRef] [PubMed]
37. Andruleviciute, V.; Makareviciene, V.; Skorupskaite, V.; Gumbyte, M. Biomass and oil content of *Chlorella* sp., *Haematococcus* sp., *Nannochloris* sp. and *Scenedesmus* sp. under mixotrophic growth conditions in the presence of technical glycerol. *J. Appl. Phycol.* **2014**, *26*, 83–90. [CrossRef]
38. Kong, W.B.; Yang, H.; Cao, Y.T.; Song, H.; Hua, S.F.; Xia, C.G. Effect of glycerol and glucose on the enhancement of biomass, lipid and soluble carbohydrate production by *Chlorella vulgaris* in mixotrophic culture. *Food Technol. Biotechnol.* **2013**, *51*, 62–69.

39. Perez-Garcia, O.; de-Bashan, L.E.; Hernandez, J.P.; Bashan, Y. Efficiency of growth and nutrient uptake from wastewater by heterotrophic, autotrophic, and mixotrophic cultivation of *Chlorella vulgaris* immobilized with azospirillum brasilense. *J. Phycol.* **2010**, *46*, 800–812. [CrossRef]
40. Gao, F.; Yang, H.L.; Li, C.; Peng, Y.Y.; Lu, M.M.; Jin, W.H.; Bao, J.J.; Guo, Y.M. Effect of organic carbon to nitrogen ratio in wastewater on growth, nutrient uptake and lipid accumulation of a mixotrophic microalgae *Chlorella* sp. *Bioresour. Technol.* **2019**, *282*, 118–124. [CrossRef]
41. Babaei, A.; Mehrnia, M.R.; Shayegan, J.; Sarrafzadeh, M.H.; Amini, E. Evaluation of nutrient removal and biomass production through mixotrophic, heterotrophic, and photoautotrophic cultivation of *Chlorella* in nitrate and ammonium wastewater. *Int. J. Environ. Res.* **2018**, *12*, 167–178. [CrossRef]
42. Massa, M.; Buono, S.; Langellotti, A.L.; Castaldo, L.; Martello, A.; Paduano, A.; Sacchi, R.; Fogliano, V. Evaluation of anaerobic digestates from different feedstocks as growth media for *Tetradesmus obliquus*, *Botryococcus braunii*, *Phaeodactylum tricornutum* and *Arthrospira maxima*. *N. Biotechnol.* **2017**, *36*, 8–16. [CrossRef]
43. Gupta, S.K.; Ansari, F.A.; Shriwastav, A.; Sahoo, N.K.; Rawat, I.; Bux, F. Dual role of *Chlorella sorokiniana* and *Scenedesmus obliquus* for comprehensive wastewater treatment and biomass production for bio-fuels. *J. Clean. Prod.* **2016**, *115*, 255–264. [CrossRef]
44. Scarponi, P.; Ghirardini, A.M.V.; Bravi, M.; Cavinato, C. Evaluation of *Chlorella vulgaris* and *Scenedesmus obliquus* growth on pretreated organic solid waste digestate. *Waste Manag.* **2021**, *119*, 235–241. [CrossRef] [PubMed]
45. Ji, M.; Yun, H.; Park, Y.; Kabra, A.N.; Oh, I.; Choi, J. Mixotrophic cultivation of a microalga *Scenedesmus obliquus* in municipal wastewater supplemented with food wastewater and flue gas CO_2 for biomass production. *J. Environ. Manag.* **2015**, *159*, 115–120. [CrossRef] [PubMed]
46. Dahmani, S.; Zerrouki, D.; Ramanna, L.; Rawat, I.; Bux, F. Cultivation of *Chlorella pyrenoidosa* in outdoor open raceway pond using domestic wastewater as medium in arid desert region. *Bioresour. Technol.* **2016**, *219*, 749–752. [CrossRef]
47. Kang, C.D.; Lee, J.S.; Park, T.H.; Sim, S.J. Complementary limiting factors of astaxanthin synthesis during photoautotrophic induction of haematococcus pluvialis: C/N ratio and light intensity. *Appl. Microbiol. Biotechnol.* **2007**, *74*, 987–994. [CrossRef] [PubMed]
48. Nzayisenga, J.C.; Eriksson, K.; Sellstedt, A. Mixotrophic and heterotrophic production of lipids and carbohydrates by a locally isolated microalga using wastewater as a growth medium. *Bioresour. Technol.* **2018**, *257*, 260–265. [CrossRef]
49. Solovchenko, A.E.; Ismagulova, T.T.; Lukyanov, A.A.; Vasilieva, S.G.; Konyukhov, I.V.; Pogosyan, S.I.; Lobakova, E.S.; Gorelova, O.A. Luxury phosphorus uptake in microalgae. *J. Appl. Phycol.* **2019**, *31*, 2755–2770. [CrossRef]
50. Feng, Y.; Li, C.; Zhang, D. Lipid production of *Chlorella vulgaris* cultured in artificial wastewater medium. *Bioresour. Technol.* **2011**, *102*, 101–105. [CrossRef]
51. Prathima Devi, M.; Venkata Subhash, G.; Venkata Mohan, S. Heterotrophic cultivation of mixed microalgae for lipid accumulation and wastewater treatment during sequential growth and starvation phases: Effect of nutrient supplementation. *Renew. Energy* **2012**, *43*, 276–283. [CrossRef]
52. Lowrey, J.; Brooks, M.S.; McGinn, P.J. Heterotrophic and mixotrophic cultivation of microalgae for biodiesel production in agricultural wastewaters and associated challenges—A critical review. *J. Appl. Phycol.* **2015**, *27*, 1485–1498. [CrossRef]
53. Markou, G. Effect of various colors of light-emitting diodes (leds) on the biomass composition of arthrospira platensis cultivated in semi-continuous mode. *Appl. Biochem. Biotechnol.* **2014**, *172*, 2758–2768. [CrossRef] [PubMed]
54. Piasecka, A.; Nawrocka, A.; Wiącek, D.; Krzemińska, I. Agro-industrial by-product in photoheterotrophic and mixotrophic culture of *Tetradesmus obliquus*: Production of ω3 and ω6 essential fatty acids with biotechnological importance. *Sci. Rep.* **2020**, *10*, 6411. [CrossRef]
55. Gomaa, M.; Ali, M.M.A. Biomass and bioenergy enhancement of microalgal biomass, lipid production and biodiesel characteristics by mixotrophic cultivation using enzymatically hydrolyzed chitin waste. *Biomass Bioenergy* **2021**, *154*, 106251. [CrossRef]
56. de Melo, R.G.; de Andrade, A.F.; Bezerra, R.P.; Correia, D.S.; de Souza, V.C.; Brasileiro-Vidal, A.C.; Viana Marques, D.d.A.; Porto, A.L.F. Chlorella vulgaris mixotrophic growth enhanced biomass productivity and reduced toxicity from agro-industrial by-products. *Chemosphere* **2018**, *204*, 344–350. [CrossRef]
57. Kadkhodaei, S.; Abbasiliasi, S.; Shun, T.J.; Fard Masoumi, H.R.; Mohamed, M.S.; Movahedi, A.; Rahim, R.; Ariff, A.B. Enhancement of protein production by microalgae *Dunaliella salina* under mixotrophic conditions using response surface methodology. *RSC Adv.* **2015**, *5*, 38141–38151. [CrossRef]
58. Heredia-Arroyo, T.; Wei, W.; Ruan, R.; Hu, B. Mixotrophic cultivation of *Chlorella vulgaris* and its potential application for the oil accumulation from non-sugar materials. *Biomass Bioenergy* **2011**, *35*, 2245–2253. [CrossRef]
59. Kong, W.; Song, H.; Cao, Y.; Yang, H.; Hua, S.; Xia, C. The characteristics of biomass production, lipid accumulation and chlorophyll biosynthesis of *Chlorella vulgaris* under mixotrophic cultivation. *Afr. J. Biotechnol.* **2011**, *10*, 11620–11630. [CrossRef]
60. Ruiz-marin, A.; Canedo-lópez, Y.; Narvaez-garcía, A.; Robles-, J.C.; Zavala-loria, J.C. Productivity and biodiesel quality of fatty acids contents from *Scenedesmus obliquus* in domestic wastewater using phototrophic and mixotrophic cultivation systems. *Open Biotechnol. J.* **2018**, *12*, 229–240. [CrossRef]
61. González-González, L.M.; De-Bashan, L.E. Toward the enhancement of microalgal metabolite production through microalgae–bacteria consortia. *Biology* **2021**, *10*, 282. [CrossRef] [PubMed]
62. FAO/WHO. *Energy and Protein*; World Health Organization & Food and Agriculture Organization of the United Nations: Geneva, Switerland, 1973.

63. Miao, M.S.; Yao, X.D.; Shu, L.; Yan, Y.J.; Wang, Z.; Li, N.; Cui, X.T.; Lin, Y.M.; Kong, Q. Mixotrophic growth and biochemical analysis of *Chlorella vulgaris* cultivated with synthetic domestic wastewater. *Int. Biodeterior. Biodegrad.* **2016**, *113*, 120–125. [CrossRef]
64. Grosse, J.; Brussaard, C.P.D.; Boschker, H.T.S. Nutrient limitation driven dynamics of amino acids and fatty acids in coastal phytoplankton. *Limnol. Oceanogr.* **2019**, *64*, 302–316. [CrossRef]
65. El-Sheekh, M.M.; Eladel, H.M.; Battah, M.G.; Abd-Elal, S.M. Effect of different nitrogen sources on growth and biochemical composition of the green microalgae *Scenedesmus obliquus* and *Chlorella kessleri*. *Int. Confer. Biol. Sci.* **2004**, *3*, 419–432.
66. Wang, Y.; Tibbetts, S.M.; Berrue, F.; McGinn, P.J.; MacQuarrie, S.P.; Puttaswamy, A.; Patelakis, S.; Schmidt, D.; Melanson, R.; MacKenzie, S.E. A rat study to evaluate the protein quality of three green microalgal species and the impact of mechanical cell wall disruption. *Foods* **2020**, *9*, 1531. [CrossRef] [PubMed]
67. FAO. *Dietary Protein Quality Evaluation in Human Nutrition: Report of an FAO Expert Consultation*; Food and Agriculture Organization: Rome, Italy, 2013.
68. Kent, M.; Welladsen, H.M.; Mangott, A.; Li, Y. Nutritional evaluation of australian microalgae as potential human health supplements. *PLoS ONE* **2015**, *10*, e0118985. [CrossRef]
69. Li, Y.R.; Tsai, W.T.; Hsu, Y.C.; Xie, M.Z.; Chen, J.J. Comparison of autotrophic and mixotrophic cultivation of green microalgal for biodiesel production. *Energy Procedia* **2014**, *52*, 371–376. [CrossRef]
70. Choi, H.J.; Yu, S.W. Influence of crude glycerol on the biomass and lipid content of microalgae. *Biotechnol. Biotechnol. Equip.* **2015**, *29*, 506–513. [CrossRef]
71. Gross, M.; Wen, Z. Yearlong evaluation of performance and durability of a pilot-scale revolving algal biofilm (rab) cultivation system. *Bioresour. Technol.* **2014**, *171*, 50–58. [CrossRef]
72. Rattanapoltee, P.; Kaewkannetra, P. Cultivation of microalga, chlorella vulgaris under different auto-hetero-mixo trophic growths as a raw material during biodiesel production and cost evaluation. *Energy* **2014**, *78*, 4–8. [CrossRef]
73. Rincon, S.M.; Romero, H.M.; Aframehr, W.M.; Beyenal, H. Biomass production in *Chlorella vulgaris* biofilm cultivated under mixotrophic growth conditions. *Algal Res.* **2017**, *26*, 153–160. [CrossRef]
74. Mahapatra, D.M.; Chanakya, H.N.; Ramachandra, T.V. Bioremediation and lipid synthesis through mixotrophic algal consortia in municipal wastewater. *Bioresour. Technol.* **2014**, *168*, 142–150. [CrossRef] [PubMed]
75. Alagawany, M.; Elnesr, S.S.; Farag, M.R.; Abd El-Hack, M.E.; Khafaga, A.F.; Taha, A.E.; Tiwari, R.; Iqbal Yatoo, M.; Bhatt, P.; Khurana, S.K.; et al. Omega-3 and Omega-6 fatty acids in poultry nutrition: Effect on production performance and health. *Animals* **2019**, *9*, 573. [CrossRef] [PubMed]
76. Martins, D.A.; Custódio, L.; Barreira, L.; Pereira, H.; Ben-Hamadou, R.; Varela, J.; Abu-Salah, K.M. Alternative sources of n-3 long-chain polyunsaturated fatty acids in marine microalgae. *Mar. Drugs* **2013**, *11*, 2259–2281. [CrossRef] [PubMed]
77. Arbib, Z.; Marín, D.; Cano, R.; Saúco, C.; Fernandez, M.; Lara, E. Chapter 9—Large-scale demonstration of microalgae-based wastewater biorefineries. In *Integrated Wastewater Management and Valorization Using Algal Cultures*; Elsevier: Amsterdam, The Netherlands, 2022; pp. 215–234.