AgriEngineering

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Bioremediation of Basil Pesto Sauce-Manufactured Wastewater by the Microalgae *Chlorella vulgaris* Beij. and *Scenedesmus* sp.

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Abstract: *Chlorella vulgaris* and *Scenedesmus* sp. are commonly used in wastewater treatment due to their fast growth rates and ability to tolerate a range of environmental conditions. This study explored the cultivation of *Chlorella vulgaris* and *Scenedesmus* sp. using wastewater from the food industry, particularly from Italian basil pesto production tanks. The experiment involved different carbon dioxide concentrations and light conditions with a dilution rate of basil pesto wastewater at 1:2. Both microalgae strains were able to grow on pesto wastewater, and biomass characterization highlighted the influence of CO_2 supply and light irradiation. The highest lipid storage was 79.3 ± 11.4 mg $g_{dry\ biomass}{}^{-1}$ and 75.5 ± 13.3 mg $g_{dry\ biomass}{}^{-1}$ for *C. vulgaris* and *S. obliquus* under red light (5% CO_2 supply) and white light (0.04% CO_2 supply), respectively. Protein storage was detected at 20.3 ± 1.0% and 24.8 ± 1.3% in *C. vulgaris* and *S. obliquus* biomasses under white light with a 5% CO_2 and 0.04% CO_2 supply, respectively. The removal of P, N, chemical oxygen demand, and biological oxygen demand resulted in 80–100%, 75–100%, 26–35%, and 0–20%, respectively.

Keywords: microalgae; *Chlorella*; *Scenedesmus*; wastewater remediation; lipid storage

Citation: Scarponi, P.; Frongia, F.; Cramarossa, M.R.; Roncaglia, F.; Arru, L.; Forti, L. Bioremediation of Basil Pesto Sauce-Manufactured Wastewater by the Microalgae *Chlorella vulgaris* Beij. and *Scenedesmus* sp. *AgriEngineering* **2024**, *6*, 1674–1682. https://doi.org/10.3390/agriengineering6020096

Academic Editors: Mehmet Başlar and Barış Yalınkılıç

Received: 17 April 2024
Revised: 3 June 2024
Accepted: 6 June 2024
Published: 12 June 2024

1. Introduction

In 2015, the United Nations General Assembly (UNGA) provided the 17 Sustainable Development Goals (SDGs) that must be achieved by 2030. Among all the goals reported by the UNGA, microalgae found a fundamental application in the resolution of Goal 6 (Clean Water and Sanitation) and Goal 7 (Affordable and Clean Energy) [1]. Microalgae integration in wastewater treatment plants and biofuel production has been studied extensively due to their strong phytoremediation effect on agro-industrial effluents and storage of secondary high-value products (e.g., lipids, starch). These secondary high-value products can be used in the synthesis of biodiesel, bioethanol, biogas, or biohydrogen [2–4]. In addition, the photosynthetic activity of microalgae has increased their application in greenhouse gas treatment and biogas upgrading processes, with potential integration as coupled-biological treatment systems [5,6]. Microalgae cultivation leads to reduced environmental pollutants, decreased plant input costs, and their integration as a biological treatment in the bio-circular green economy concept [7–9]. To sustain large-scale microalgae biorefinery processes, an economically viable carbon source is essential. Agricultural residues and by-products from the food industry could provide low-cost nutrient input for cost-effective and environmentally sustainable microalgae-based wastewater treatment. The estimated agro-waste production in Europe is about 250 million tons per year, consisting of damaged fruits, unmarketable products that do not meet qualitative standards, unripe produce, and wastewater. These wastes have a high organic matter concentration, creating an

environmental problem for their disposal [10]. However, agro-waste chemical composition could support microalgae cultivation [11]. Recently, seasonal food waste treatment using microalgae has been explored with wine lees from wine production or its digestate [12,13] and olive oil production [14]. The results showed promising applications for integrating this biological treatment in loco for small food producers. *Chlorella vulgaris* and *Scenedesmus* sp. represent an excellent choice for microalgae cultivation, known for their ability to fix CO_2 and remove nutrients from wastewater [7,13].

Among all agro-productions in Italy, one of the most popular traditional sauces produced and consumed worldwide is the "basil pesto sauce". Basil pesto sauce is made from fresh basil leaves, pine nuts, garlic, cheese, and extra virgin olive oil. Basil, the main ingredient, is a herbaceous plant rich in phenolic compounds, tannins, alkaloids, flavonoids, and saponins [15]. The industrial production of basil pesto has a significant economic impact in specific Italian regions (e.g., Liguria), and there is currently a lack of knowledge regarding its wastewater treatment.

This research aimed to identify the growth parameters of *Chlorella vulgaris* and *Scenedesmus* sp. using different concentrations of basil pesto sauce-manufactured wastewater. To achieve this goal, the optimization of cultivation conditions was carried out by varying the percentage of wastewater and CO_2, as well as light irradiation. A comparative analysis of biomass responses (growth rate, lipid productivity, CO_2 fixation rates) was conducted to understand the potential integration of in loco microalgae cultivation in basil pesto sauce manufacturing.

2. Materials and Methods

2.1. Experimental Conditions

The microalgae strains *Chlorella vulgaris* Beij. 863 and *Scenedesmus* sp. 329, obtained from the ACUF algal collection at the Department of Biology of the University of Federico II of Naples (Italy), were cultured in Bold's Basal Medium (BBM) supplemented with vitamins, following the protocol described in Bischof et al. [16] and Starr et al. [17]. Inoculum cultures were maintained under continuous mechanical agitation at 80 rpm using a horizontal shaker (Universal Table Shaker 709, Lab Supply, Fattoruso Tech SRL, Italy) under continuous illumination of 50 µE at room temperature (25 °C).

2.2. Evaluation of Pesto Wastewater as Substrate for Microalgae Cultivation

The particulate residues from wastewater were removed through filtration with a paper filter as a pretreatment. The liquid fraction, without sterilization, was tested as a substrate for *Chlorella* and *Scenedesmus* sp. cultivation. The chemical characterization of basil pesto wastewater is reported in Table 1.

Table 1. Chemical characterization of basil pesto wastewater.

Total phosphorus, P (mg L^{-1})	3.67 ± 0.63	[18,19]
Total nitrogen, N (mg L^{-1})	4.21 ± 0.67	[20]
Chemical oxygen demand, COD (mg $L_{O2}{}^{-1}$)	245 ± 12	[21]
Biochemical oxygen demand, BOD (mg $L_{O2}{}^{-1}$)	86 ± 3	[22]

An inoculum concentration of 1×10^6 cell mL^{-1} was applied for both microalgae strains under the experimental conditions, and the basil pesto wastewater was tested at different concentrations: 25%, 50%, 75%, and 100% *v*/*v* (wastewater/BBM medium). After one day of microalgae adaptation in orbital flasks, the experiments were conducted in parallel using a multi-photobioreactor system (Multi-Cultivator MC 1000-OD, PSI-CZ Drásov 470, Pribram, Czech Republic). Light irradiation was set up using warm white (WW) (2700 K) or deep red (red) (660 nm) light at 100 µE. The photoperiod was configured at 8:16 h dark/light, selected to mimic the summer period of the basil plant harvest and basil pesto production. All conditions were at room temperature with continuous air insufflation (flow rate 0.8 L min^{-1}). Microalgae growth was monitored for seven days, and the experiment was conducted in triplicate. The results are reported as mean values with standard deviations.

The data obtained (results not reported) highlighted that the experimental condition with 50% *v*/*v* (dilution rate 1:2) of pesto wastewater was the most promising substrate for microalgae cultivation in subsequent experiments.

2.3. Evaluation of 50% v/v Pesto Wastewater and CO_2 Addition

The experiment upgrade involved the addition of CO_2 at different percentages: 0.04%, 2%, and 5%. The inoculum concentration was 1×10^6 cell mL^{-1}, and the experiment was carried out under a light intensity of 100 µE with an 8:16 dark/light photoperiod at room temperature and a gas flow rate of 0.8 L m^{-1}. The experiment was carried out in batch conditions for seven days using the Multi-Cultivator MC 1000-OD.

2.4. Monitoring Analyses and Biomass Storage

Daily, the Multi-Cultivator MC 1000-OD was used to record the absorption data at 720 nm of wavelength. Biomass quantification at the end of the test was carried out gravimetrically. Daily biomass samples were collected, centrifugated (NEYA 16 high speed, Carpi, Italy) at 4500 rpm for 20 min, frozen at −80 °C, and lyophilized (HETO Lyolab 3000, Thermo Fisher Scientific, Waltham, MA, USA) for chemical characterization analyses.

2.4.1. Elementary Analysis

Lyophilized biomass samples were analyzed using the Flash 2000 CHNS Analyser (Thermo Fisher Scientific) to quantify the organication of CO_2. The carbon dioxide fixation rate (PCO_2) was calculated following Equation (1) [23]:

$$\boldsymbol{PCO_2 = Cc \times P \times \left(\frac{MCO_2}{MC}\right)} \quad (1)$$

where Cc represents the average carbon content in the dry biomass according to the elemental analysis, P (g^{-1} L^{-1} mol^{-1}) is the microalgae biomass productivity, MCO_2 is the molecular weight of CO_2, and MC is the molecular weight of carbon.

The concentration of proteins in the microalgae biomass was calculated following Equation (2) [24].

$$\boldsymbol{Crude\ protein(\%) = N(\%) \times 6.25} \quad (2)$$

The specific growth rate (μ_{max}) was calculated based on OD 720 nm data following Equation (3).

$$\boldsymbol{\mu_{max} = ln\left(\frac{N_t - N_0}{t_t - t_0}\right)} \quad (3)$$

$N_t - N_0$ and $t_t - t_0$ were the OD values on day zero and the final day, respectively.

Division day and generation time were calculated following Equations (4) and (5) [25].

$$\boldsymbol{Div.d.(day) = \frac{\mu_{max}}{ln2}} \quad (4)$$

$$\boldsymbol{Generation\ time = \frac{1}{Div.d.}} \quad (5)$$

2.4.2. Lipids Extraction

Lipid extraction was carried out using hexane (Merk, New Jersey, NJ, USA) as an organic solvent, following the Blight et Dyer method [26]. The extraction process used the ultrasonic-assisted procedure (UP200St, 200 W, 26 kHz, Hielscher, Teltow, Germany). Specifically, 0.2 g of lyophilized biomass was suspended in 2 mL of water and sonicated (25 W, width 50%) for 4 min on ice. Subsequently, the samples were centrifugated at 4500 rpm for 20 min, and 2 mL of hexane was added to the liquid fractions. After hexane addition, the samples were vortexed for 30 s and then centrifugated at 450 rpm for 20 min. Quantification of the extracted lipids was performed gravimetrically after evaporation of

the solvent using a rotary evaporator (Concentrator 5301, Eppendorf AG 22331 Hamburg Germany) for 30 min.

3. Results and Discussion

3.1. Wastewater–CO_2 Combined Experiments

Pesto wastewater, when used as a substrate for microalgae cultivation, exhibited promising biomass production, particularly when applied with a low dilution ratio (50% *v*/*v*). OD monitoring of *Chlorella* and *Scenedesmus* (Figure 1) revealed a similar growth trend for both microalgae strains. *Chlorella* biomass development was strongly influenced by red light irradiation compared to *Scenedesmus*. The combination of red light irradiation and CO_2 supply showed effects on the exponential growth phase, resulting in a decrease in the lag phase (Figure 1b,c,e,f). Similar trends in OD measurements were identified by He et al. [27], where different degrees of light irradiation had varying effects on *Chlorella* and *Scenedesmus* growth in wastewater. As reported by He et al. [27] and Liu et al. [28], 680–690 nm OD wavelengths were used to monitor biomass development, corresponding to the wavelengths of maximum chlorophyll absorption. However, in this study, the use of a 720 nm wavelength for OD measurement could not be compared with other literature data. Typically, OD analysis conducted at 680–690 nm may not consider the increase or decrease in chlorophyll content associated with the physiological microalgal response to environmental conditions. For this reason, using 680–690 nm for microalgae monitoring could over- or under-estimate microalgae biomass production [29,30]. Wavelengths higher than 700 nm did not affect OD measurements, as the antenna system did not absorb wavelengths greater than 700 nm [30].

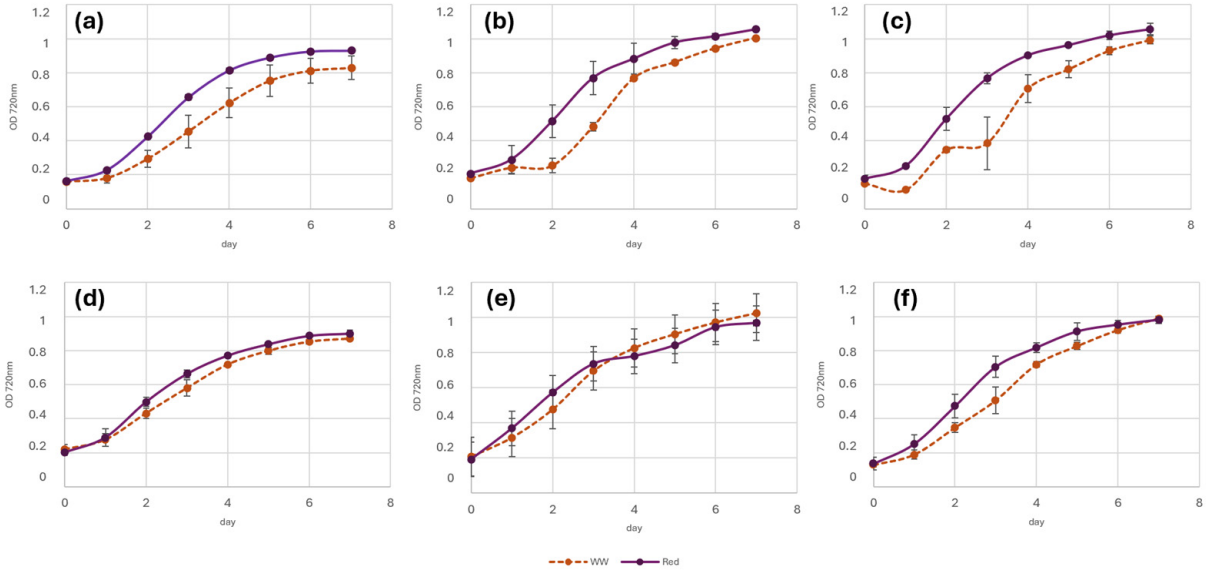

Figure 1. OD 720 nm data for experimental conditions: *Chlorella* 50% *v*/*v* wastewater with 0.04% CO_2 (**a**), 2% CO_2 (**b**,**c**), and 5% CO_2 (**c**); *Scenedesmus* 50% *v*/*v* wastewater with 0.04% CO_2 (**d**), 2% CO_2 (**e**), and 5% CO_2 (**f**).

The quantification of dry weight, lipid, and protein storage (Table 2) highlighted the influence of WW and red light on biomass production and macromolecular storage in both microalgae strains cultivated under a normal CO_2 air supply (0.04%). Red light positively influenced biomass production and lipid storage in both microalgae strains, although the highest protein storage was observed during cultivation under WW light. The 2% and 5% CO_2 supply conditions showed similar biomass productivity and lipid storage. Conversely, a trend of higher protein storage under WW light was identified for both strains. However, biomass production results were consistent with findings from other literature data [27,31,32]. The maximum lipid productivity (Figure 2e,f and Table 2) and protein storage (Figure 2g,h and Table 2) were detected

in *Chlorella* and *Scenedesmus* biomasses under WW light irradiation with a 0.04% CO_2 supply: 153.4 ± 11.4 mg $g_{dry\ biomass}{}^{-1}$ d^{-1} and 75.5 ± 13.3 mg $g_{dry\ biomass}{}^{-1}$ d^{-1} lipid productivity and 23.14 ± 3.41% and 24.82 ± 1.25% protein storage for *Chlorella* and *Scenedesmus*, respectively. These results contrasted with literature data, where red light irradiation at 5000 lux using a photoperiod of 12:12 was found to increase photosystem II activity and influence biomass production and lipid storage, with an accumulation higher than 70% in *Chlorella* and *Scenedesmus* strains [27]. In addition, the protein percentage detected during the test indicated a shift in the metabolic synthesis pathway, where nitrogen from wastewater typically increases protein storage in microalgae biomass, as reported by Wang et al. [33]. The discrepancy in macromolecular storage observed when comparing the results with the literature data may be correlated with differences in the experimental setup such as light irradiation, photoperiod, wastewater composition, and CO_2 supply. Indeed, as reported by [27], the different degrees of light irradiation, photoperiods, and substrates significantly influenced the metabolic synthesis pathway of *Chlorella* and *Scenedesmus* strains. Evidence of these influences can be seen in the growth rate (μ_{max}), division per day, and generation time comparison (Table 2) with the literature data. As reported by Ajala et al. [34], Wang et al. [33], and Singh et al. [35], the cultivation of *Chlorella* and *Scenedesmus* on different wastewater samples showed μ_{max} values ranging from 0.05 to 0.39 d^{-1} and 0.07 to 0.20 d^{-1}, respectively. The application of pesto wastewater with a low N concentration and high COD value linked with different degrees of light irradiation and CO_2 supply likely positively influenced biomass production by enhancing photosynthetic activity and lipid storage in *Chlorella* and *Scenedesmus* strains.

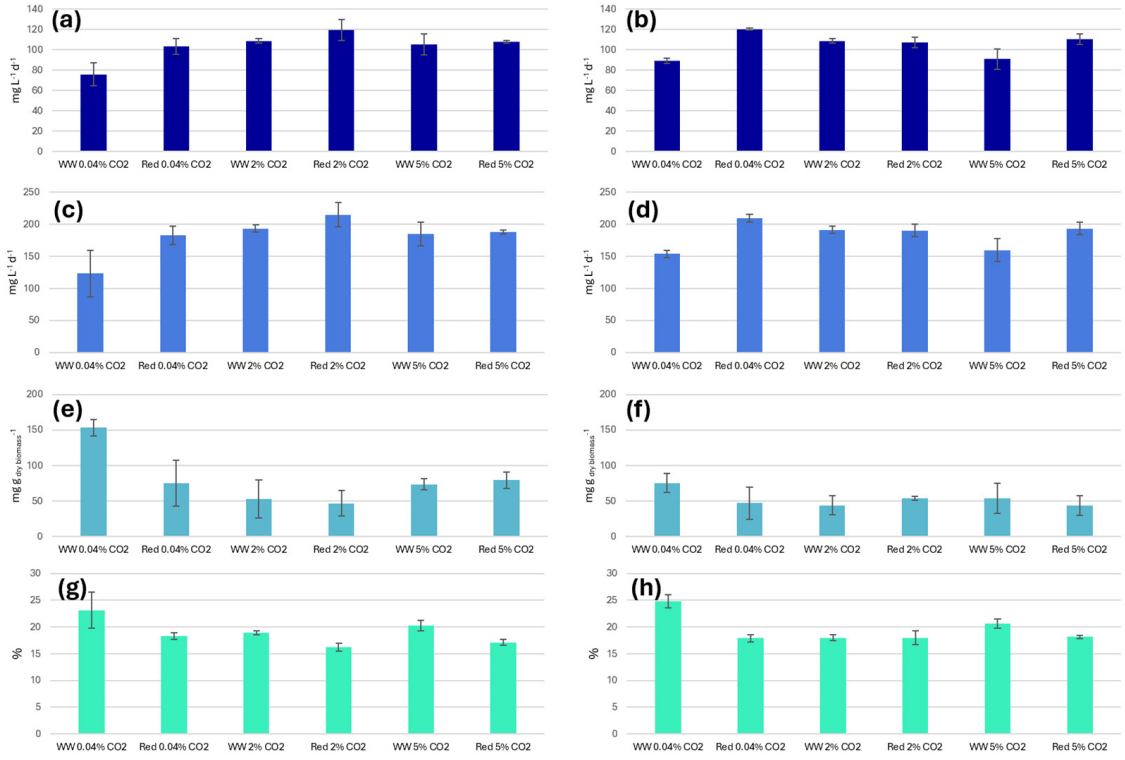

Figure 2. Dry weight productivity (mg L^{-1} d^{-1}) for (**a**) *Chlorella* 50% *v*/*v* wastewater and (**b**) *Scenedesmus* 50% *v*/*v* wastewater experimental conditions; PCO_2 (mg L^{-1} d^{-1}) for (**c**) *Chlorella* 50% *v*/*v* wastewater and (**d**) *Scenedesmus* 50% *v*/*v* wastewater experimental conditions; lipid storage (mg $g_{dry\ biomass}{}^{-1}$) for (**e**) *Chlorella* 50% *v*/*v* wastewater and (**f**) *Scenedesmus* 50% *v*/*v* wastewater experimental conditions; and protein storage (%) for (**g**) *Chlorella* 50% *v*/*v* wastewater and (**h**) *Scenedesmus* 50% *v*/*v* wastewater experimental conditions.

Table 2. Biomass production, carbon dioxide fixation rate (PCO_2), protein and lipid storage, growth rate (μ_{max}), division per day, and generation time during *C. vulgaris* and *Scenedesmus* sp. cultivation with basil pesto wastewater 50% *v*/*v* with the addition of 0.004%, 2%, and 5% CO_2.

		CO_2 (%)	Light	Dry Weight (mg L^{-1} d^{-1})	PCO_2 (mg L^{-1} d^{-1})	Crude Protein (%)	Lipid (mg g $_{dry\ biomass}^{-1}$)	μ_{max} (d^{-1})	Divisions per Day	Generation Time (d)
Chlorella	50% *v*/*v* basil pesto wastewater	0.04	White	75.9 ± 11.4	123.1 ± 36.4	23.1 ± 3.4	53.4 ± 11.4	0.5 ± 0.0	0.7 ± 0.0	1.5 ± 0.1
			Red	103.6 ± 7.6	182.9 ± 13.9	18.3 ± 0.6	75.1 ± 32.2	0.5 ± 0.0	0.8 ± 0.0	1.3 ± 0.0
		2	White	108.9 ± 2.5	193.3 ± 5.4	18.9 ± 0.4	52.9 ± 26.6	0.6 ± 0.1	0.8 ± 0.1	1.3 ± 0.2
			Red	119.6 ± 10.1	214.6 ± 18.9	16.2 ± 0.7	46.6 ± 18.0	0.5 ± 0.1	0.7 ± 0.1	1.4 ± 0.2
		5	White	105.4 ± 10.1	185.1 ± 18.5	20.3 ± 1.0	73.5 ± 8.0	0.7 ± 0.0	1.1 ± 0.0	1.0 ± 0.0
			Red	108.0 ± 1.3	187.8 ± 3.1	17.1 ± 0.6	79.3 ± 11.4	0.6 ± 0.0	0.8 ± 0.1	1.2 ± 0.1
Scenedesmus		0.04	White	89.3 ± 2.5	154.1 ± 5.6	24.8 ± 1.3	75.5 ± 13.3	0.4 ± 0.0	0.5 ± 0.0	1.9 ± 0.1
			Red	120.5 ± 1.3	209.7 ± 6.2	17.9 ± 0.7	47.2 ± 22.8	0.4 ± 0.1	0.6 ± 0.1	1.7 ± 0.3
		2	White	108.9 ± 2.5	191.5 ± 5.8	18.0 ± 0.5	44.1 ± 13.6	0.4 ± 0.0	0.6 ± 0.0	1.7 ± 0.0
			Red	107.1 ± 5.1	190.3 ± 9.6	18.0 ± 1.3	53.7 ± 2.6	0.6 ± 0.1	0.8 ± 0.1	1.3 ± 0.2
		5	White	91.1 ± 10.1	159.7 ± 18.3	20.7 ± 0.8	54.1 ± 21.3	0.5 ± 0.0	0.7 ± 0.0	1.4 ± 0.0
			Red	110.7 ± 5.1	193.4 ± 9.4	18.1 ± 0.3	43.6 ± 14.2	0.6 ± 0.1	0.9 ± 0.1	1.1 ± 0.1

Biomass productivity (Figure 2a,b and Table 3) showed the red light influence on *Chlorella* and *Scenedesmus* biomass production. CO_2 supply and red light positively influenced biomass productivity for both microalgae strains.

Table 3. Concentration limit for P, ammonia, N, COD, and BOD admitted by D. Lgs 152/06 for the environmental release.

	Surface Water Discharge	Sewerage System Discharge	Ground Discharge
P (mg L^{-1})	10	10	2
NH_4^+ (mg L^{-1})	15	30	-
N (mg L^{-1})	-	-	15
COD (mg L_{O2}^{-1})	40	250	20
BOD (mg L_{O2}^{-1})	160	500	100

For *Chlorella*, the highest biomass productivity (119.6 ± 10.1 mg L^{-1} d^{-1}) was recorded under red light with a 2% CO_2 supply; for *Scenedesmus*, the highest biomass productivity (120.5 ± 1.3 mg L^{-1} d^{-1}) was achieved under red light with a 0.04% CO_2 supply. These data are consistent with the biomass productivity detected for *Chlorella* using 25% swine wastewater (0.155 g L^{-1} d^{-1}) [36]. Carbon dioxide fixation rate (Figure 2c,d and Table 3) followed the biomass productivity trend, with the highest PCO_2 detected under experimental conditions with red light irradiation and a 2% or 0.04% CO_2 supply for *Chlorella* and *Scenedesmus*, respectively. These results could inform future studies aimed at upgrading wastewater treatment processes using microalgae in loco at pesto factories.

3.2. Pesto Wastewater Phytoremediation

Chlorella and *Scenedesmus* phytoremediation effects were evaluated to determine the feasibility of applying the liquid fraction output as irrigation water or releasing it into the soil and water environment. To achieve this purpose, the chemical characterization of the effluent needed to comply with the legal limits reported by D. Lgs 152/06 (Table 3) [37].

At the end of the batch tests, chemical analysis of the liquid fraction output (Table 4) was conducted in line with D. Lgs 152/06 for discharge into the sewerage system. Significant N removal detected during the tests may be associated with a combination of gas stripping and biomass consumption for protein synthesis. The highest COD, BOD, and phosphorous removal rates were observed when *Chlorella* and *Scenedesmus* were cultivated with a 2% CO_2 supply. These data align with previous research, where microalgae cultivation on digestate resulted in nitrogen, phosphorus, and COD removal rates of 75.7–82.5%, 62.5–74.7%, and 27.4–77.8%, respectively [38,39].

Table 4. Chemical characterization of liquid effluent after *C. vulgaris* and *Scenedesmus* sp. cultivation with basil pesto wastewater 50% *v*/*v* with the addition of 0.004%, 2%, and 5% CO_2.

		Experimental Conditions		Residual Concentration in the Liquid Fraction				Removal (%)			
		CO_2 (%)	Light	P (mg L^{-1})	N (mg L^{-1})	COD (mg $L_{O2}{}^{-1}$)	BOD (mg $L_{O2}{}^{-1}$)	P	N	COD	BOD
Chlorella	50% *v*/*v* pesto wastewater	0.04	White	1.4 ± 0.1	22.5 ± 1.4	90.0 ± 4.2	32.0 ± 2.0	94.8	99.6	26.5	25.6
			Red	2.1 ± 0.1	22.5 ± 1.4	88.5 ± 3.9	32.0 ± 2.0	92.2	99.5	27.8	25.6
		2	White	0.8 ± 0.0	22.5 ± 1.3	89.5 ± 4.3	32.0 ± 1.0	97.1	99.5	26.9	25.6
			Red	2.0 ± 0.1	22.6 ± 1.4	86.5 ± 3.7	32.0 ± 2.0	92.4	99.8	29.4	25.6
		5	White	1.6 ± 0.1	22.6 ± 1.4	83.0 ± 3.3	30.5 ± 1.4	93.9	100	32.2	29.1
			Red	3.1 ± 0.2	18.7 ± 1.3	75.3 ± 2.9	28.0 ± 1.0	88.3	82.7	38.8	34.9
Scenedesmus		0.04	White	0.0 ± 0.0	19.8 ± 1.3	90.2 ± 3.1	32.0 ± 2.0	99.9	87.4	26.5	25.6
			Red	0.9 ± 0.1	22.6 ± 1.4	88.2 ± 3.1	31.5 ± 1.6	96.5	99.7	28.2	26.8
		2	White	2.4 ± 0.2	22.6 ± 1.3	87.0 ± 3.7	32.0 ± 1.0	91.1	100	29.0	25.6
			Red	2.3 ± 0.2	22.6 ± 1.4	83.5 ± 3.1	31.0 ± 2.0	91.5	99.8	31.8	27.9
		5	White	3.9 ± 0.3	22.4 ± 1.4	84.4 ± 4.0	31.0 ± 1.0	85.3	98.8	31.4	27.9
			Red	4.2 ± 0.4	18.0 ± 1.2	79.2 ± 2.8	29.0 ± 1.0	84.2	79.6	35.5	32.7

4. Conclusions

The present research aimed to optimize the potential of microalgae-based biorefineries and circular bioeconomies in the context of bioenergy production from renewable sources. The use of microalgae and photobioreactors, such as MC 1000-OD, for wastewater treatment has proven effective in reducing the demand for nitrogen, phosphorus, and chemical and biochemical oxygen demand components that characterize wastewater and represent pollutants that are harmful to the environment. The experimental findings suggest that the reduction of these substances can be optimized by controlling variables that are beneficial for microalgal growth, such as light and CO_2, thereby facilitating the capture of industrial waste. The sequestration of excess CO_2 from the atmosphere is one of the main contributions to mitigating the adverse environmental impacts of human activities. The potential benefits of this approach are twofold: the generation of purified water and microalgal biomass, which can serve various purposes, including energy production through lipid extraction.

Author Contributions: Conceptualization, P.S., L.A. and L.F.; Investigation, F.F. and M.R.C.; Data curation, P.S. and F.R.; Writing—original draft preparation, P.S. and F.F.; Writing—review and editing, P.S. and L.F.; Supervision, L.F.; Funding acquisition, L.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was made possible through funding from the European Union-Next Generation EU, facilitated by the Ministero dell'Università e della Ricerca (MUR), PRIN 2022, under the project titled 'Biotechnological synthesis of valuable Lipids and fatty acid derivatives from Agro-food industrial Residues' (BioLAR), grant number 20223E9C8S. Additionally, support was provided by a grant from the University of Modena and Reggio Emilia, FAR2021 Dipartimentale.

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

Acknowledgments: Special recognition goes to the Algal Collection University Federico II (ACUF), Naples, Italy, for providing the algal strains essential for this study. The authors also acknowledge BIOKIM SRL (Modena, Italy) for providing basil pesto sauce wastewater, a key component in the experimental setup.

Conflicts of Interest: The authors declare no conflicts of interest.

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