applied sciences

MDPI

Article

Sustainable Cultivation of *Galdieria phlegrea* in an IoT-Integrated Twin-Layer Photobioreactor: System Design, Growth Dynamics, and Isotopic Perspective

Maria Rosa di Cicco [1,*], Simona Altieri [1], Antonio Spagnuolo [2], Claudia Ciniglia [1], Chiara Germinario [3], Silvio Bove [4], Antonio Masiello [1], Carmela Vetromile [1,5], Iolanda Galante [1] and Carmine Lubritto [1,6]

[1] Dipartimento di Scienze e Tecnologie Ambientali, Biologiche e Farmaceutiche, Università degli Studi della Campania Luigi Vanvitelli, Via Vivaldi, 43, 81100 Caserta, Italy; simona.altieri@unicampania.it (S.A.); claudia.ciniglia@unicampania.it (C.C.); antonio.masiello@unicampania.it (A.M.); carmela.vetromile@unicampania.it (C.V.); iolanda.galante@studenti.unicampania.it (I.G.); carmine.lubritto@unicampania.it (C.L.)
[2] Energreenup S.r.l., Via Acquaviva 192, 81100 Caserta, Italy; a.spagnuolo@energreenup.it
[3] Dipartimento di Scienze e Tecnologie, Università degli Studi del Sannio, Via Dei Mulini 59/a, 82100 Benevento, Italy; chiara.germinario@unisannio.it
[4] Laboratoire de Mathématiques Raphaël Salem, UMR 6085 CNRS, Université de Rouen, Avenue de l'Université, B.P. 12, Technopôle du Madrillet, 76801 Saint-Étienne-du-Rouvray, France; silvio.bove@univ-rouen.fr
[5] Reverseup S.r.l., Via Francesco Napoli, 8F, 84081 Baronissi, Italy
[6] INFN Sezione di Napoli, Complesso di Monte Sant'Angelo, Strada Comunale Cinthia, 80126 Napoli, Italy
* Correspondence: mariarosa.dicicco@unicampania.it

Abstract: This study showcases an attached-biomass system based on twin-layer technology for cultivating *Galdieria phlegrea* using municipal wastewater, equipped with a smart sensor system for the remote monitoring of operational parameters. From an industrial scale-up perspective, the system offers high scalability, with low impact and operating costs. Mathematical approximation modelling identified the optimal growth conditions across five experiments. The theoretical yield was estimated to reach 1 kg_{DW}/m^2 of biomass within two months. Integrated use of isotopic mass spectrometry and spectrophotometric methods allowed us to study the metabolic strategies implemented by the algal community during the best growth condition at different resolutions, showing an increase in the nitrogen concentration over time and a favourable affinity of the organism for nitrogen species that are commonly present in the urban effluent. SEM studies showed a clean algal biofilm (free of foreign organisms), which could guarantee usage in the high economic potential market of biorefineries.

Keywords: attached biomass; *Galdieria phlegrea*; EA-IRMS; SEM; smart monitoring; stable isotopes; twin layers; urban wastewater

Academic Editor: Juan García Rodríguez

Received: 15 April 2025
Revised: 3 May 2025
Accepted: 6 May 2025
Published: 7 May 2025

Citation: di Cicco, M.R.; Altieri, S.; Spagnuolo, A.; Ciniglia, C.; Germinario, C.; Bove, S.; Masiello, A.; Vetromile, C.; Galante, I.; Lubritto, C. Sustainable Cultivation of *Galdieria phlegrea* in an IoT-Integrated Twin-Layer Photobioreactor: System Design, Growth Dynamics, and Isotopic Perspective. *Appl. Sci.* **2025**, *15*, 5220. https://doi.org/10.3390/app15095220

1. Introduction

Microalgae possess significant and multifaceted biotechnological potential due to their photosynthetic and CO_2-fixing abilities, as well as their production of valuable compounds such as fatty acids, proteins, and carbohydrates [1]. Their applications range from the food industry, where they are used for tailored nutritional supplements [2], to applications in the energy sector, contributing to the production of biofuels and bio-hydrogen [3], as well as agriculture, where they serve as a source for biofertiliser production [4]. Moreover, their ability to adsorb ionic species from liquid matrices makes microalgae valuable for

environmental remediation and pioneering applications in biomining and urban mining, including the recovery of critical raw materials from electronic waste [5,6].

The industrial-scale production of microalgae began in the early 1960s and has since gained increasing traction, with an expanding number of algal strains now regulated and commercially cultivated (Novel Food status Catalogue published and available on the website https://ec.europa.eu/food/food-feed-portal/screen/novel-food-catalogue/search—accessed on 28 February 2025). The most commercially widespread phyla and classes are those with the most desirable nutritional or energy profiles, particularly for food-related applications [7]. Currently, the commercial landscape is dominated by green and blue-green algae, particularly *Chlorella* spp. and *Limnospira/Arthrospira* spp. [8]. However, in recent years, the scientific community has increasingly recognised the biotechnological potential and diverse applications of extremophilic strains [9].

Among these, Cyanidiophyceae—and, in particular, the genus *Galdieria*—hold unique biotechnological interest due to their tolerance and metabolic productivity under environmental conditions that are typically inhospitable to most eukaryotes. These conditions include highly acidic pH, elevated temperatures (above 50 °C), and especially the presence of typically phytotoxic elements in their surroundings [10,11]. When considered within a biorefinery framework, these extremophilic strains can be efficiently cultivated using municipal effluents as a nutrient source, offering significant advantages. Specifically, their ability to utilise nitrogen- and phosphorus-rich waste compounds reduces cultivation costs by minimising the need for synthetic culture media. At the same time, by assimilating these compounds for growth, microalgae contribute to the phytoremediation of polluted effluents [12]. Furthermore, the use of wastewater as a cultivation medium decreases the reliance on freshwater and marine water resources, which in Europe account for approximately 96% of the total water usage in this sector [8].

Despite these advantages, the cultivation of *Galdieria* species using municipal effluents remains largely unexplored in the international literature [13]. To date, only two strains have been studied: *G. sulphuraria* CCMEE 5587.1 and *G. phlegrea* ACUF 784.3. In the first case, *G. sulphuraria* was cultivated using municipal wastewater from the Las Cruces wastewater treatment plant (New Mexico, USA) in semi-continuous plastic bag type photobioreactors (PBRs), to develop a process suited for hot, arid regions [14]. The second study focused on *G. phlegrea*, which was cultivated in urban wastewater from the province of Salerno (Southern Italy) using laboratory-scale cultivation methods [15]. This strain was specifically selected and studied in comparison with *G. sulphuraria* to assess its potential for growth in milder climatic conditions compared to New Mexico.

Scaling up these suspended cultivation methods to industrial scale poses significant technical challenges, regardless of which microalgal species has been selected. The management of culture conditions, such as temperature, pH, nutrient availability and light exposure, is critical to maximise biomass production [16]. In addition, the collection and separation of biomass from liquid phase reactors can be complex and, above all, costly. Traditional methods such as centrifugation and filtration can significantly affect biomass production costs, accounting for between 20% and 60% of total costs [17,18]. Nevertheless, the economic sustainability of microalgae cultivation also relies on profits deriving from the sale of biomass or bioproducts, which are usually expected to be higher with purer biomass [19].

Therefore, optimising existing cultivation processes and integrating innovative technologies are essential to making microalgae production competitive in commercial markets. In this context, twin-layer technology, recently introduced in the scientific community, has proven effective for cultivating *G. sulphuraria* and other microalgae using both standard

culture media and secondary urban effluents [4,20,21]. Beyond enhancing cultivation efficiency, this system addresses many of the operational limitations previously discussed.

When considering the scalability of cultivation systems, one critical aspect is the implementation of efficient management strategies. In large-scale applications—such as wastewater treatment plants, often located far from urban areas and research facilities—remote monitoring of system performance becomes essential [22]. Smart monitoring technologies allow operators to track system conditions in real time and intervene only when necessary, such as in cases of malfunction or biomass harvesting, thereby optimising maintenance and operational efforts [23].

Within this framework, the present study aims to address current challenges in sustainable microalgae cultivation by developing an attached-biomass photobioreactor designed for the growth of *G. phlegrea*, using urban wastewater as a nutrient source. The system integrates a platform based on IoT (Internet of Things) to enable continuous, remote monitoring of environmental parameters, contributing to enhanced process control and scalability.

In addition to the engineering innovation, the study introduces a robust analytical approach by combining conventional spectrophotometric methods with high-resolution techniques such as EA-IRMS (Elemental Analysis–Isotope Ratio Mass Spectrometry) and SEM-EDS (Scanning Electron Microscopy–Energy-Dispersive Spectroscopy). This integrated methodology enables the tracking of carbon and nitrogen concentrations in the biomass, along with their isotopic compositions ($\delta^{13}C$ and $\delta^{15}N$), offering valuable insights into nutrient assimilation and the metabolic responses of *G. phlegrea* under different cultivation conditions.

The structure of the manuscript reflects this dual focus on system design and experimental validation. It first outlines the scientific and technical rationale behind the selection of twin-layer technology for the photobioreactor configuration, emphasising its advantages for surface-adhered cultivation and compatibility with wastewater-based media. The subsequent section provides a detailed description of the physical layout and operating principles of the prototype, highlighting the integration of the IoT-based monitoring system and its capacity to measure key environmental variables such as temperature, pH, and light intensity in real time.

This is followed by a presentation of the cultivation experiments, which were designed to evaluate the performance of *G. phlegrea* under varying environmental conditions. Each test involved controlled modifications of individual parameters—namely, temperature, nutrient concentrations, and light exposure—to assess their impact on biomass growth. Particular attention is devoted to the physiological and metabolic responses of the microalga, as characterised through the combined use of spectrophotometric, isotopic, and microscopic analyses. The quantitative tracking of C and N dynamics, along with isotopic profiling ($\delta^{13}C$ and $\delta^{15}N$), enables a deeper understanding of nutrient uptake mechanisms and carbon fixation strategies specific to this extremophilic species.

This integrated approach provides a valuable step towards a scalable and sustainable bioprocessing platform. By combining innovative reactor engineering with advanced analytical tools and real-time process monitoring, the study contributes to overcoming key limitations in the industrial deployment of microalgal systems for bioresource production and wastewater valorisation.

Rationale Behind the Selection of Twin-Layer Cultivation Technology

The cultivation system implemented and used in this study is based on the operating principles of twin-layer type photobioreactors (TL-PBRs). These attached-biomass systems are based on the presence of two layers, namely, (i) a macroporous layer, which is traditionally referred to as the “source layer” and is the one through which the culture medium

flows; and (ii) a microporous layer, which is conventionally referred to as the "substrate layer" and is the one onto which the biomass is attached [4,21].

The first important advantage of this technology is the possibility offered by this system to safely use municipal wastewater as a microalgal growing medium at zero cost, which is an extremely advantageous condition from the point of view of process impacts. In fact, the presence of a microporous substrate guarantees that only the necessary nutrients for the organisms are able to pass through it, leaving the biomass isolated from the ensemble of microparticles and/or any micro-aggregates present in the municipal sewage, as well as competitor microorganisms [4]. As a result, the processed output should be a clean algal biomass devoid of extraneous aggregates that would invalidate its purity, which will provide market value benefits when produced for future applications in biotechnological sectors. This practical advantage becomes even more relevant and critical when dealing with primary effluents that have not yet undergone activated sludge oxidation treatment, as in the case of the present study (see Section 2.3). Such effluents are indeed full of potential because they are rich in their original nutrients that have not yet been removed/processed by activated sludge, but they also have higher turbidity levels due to the lack of treatment [24]. Because of this, when the intention is to cultivate microalgae in liquid phase PBR using municipal effluents (or other types of effluents), scientific evidences often confront stakeholders with a choice: (a) if the priority is to obtain higher-value clean biomass, then it shall be necessary to select less turbid (cleaner) effluents or to perform complex pre-treatments on the effluent before its contact with the biomass for cultivation, thus affecting the process performance [25]; (b) if the priority is achieving only a quantitatively high-performance cultivation, then it might be necessary to focus on other aspects of the cultivation system and this might affect the purity of the biomass and, consequently, also its sale value [18]. The system presented here makes it possible to avoid this choice and obtain a clean biomass with a low energy/material demand.

The second important advantage offered by these systems is the possibility of cultivating the algal biomass in the form of a dense, low moisture algal paste, which can be easily harvested using spatulas (manually) or low-intensity air sprays (indirect harvesting). This last solution was proposed by [21], when hypothesising a method for more frequent and abundant harvesting on an industrial scale. Regarding this advantage, species belonging to the genus *Galdieria* (as in this case) claim the advantage of typically developing and thriving in nature as algal mats on mineral substrates [26], thereby being able to (i) recover moisture and macro/micro nutrients through contact with the substrate on which they grow, and (ii) exchange gases directly through the interface in contact with the air. Consequently, the adoption of TL systems might prove to be a strategically successful choice for the cultivation of these valuable extremophilic microorganisms.

Gathering the above considerations, the last important advantage consists of the overall energy efficiency of the system. While liquid-phase cultures require stirring systems that are almost always electromechanical to ensure continuous mixing [19], the only units that can be accountable for appreciable energy consumption with this system are the pump required for the recirculation of the culture media (as explained in detail in Section 2.1) and the monitoring system. Moreover, this TL cultivation system eliminates the intermediate stages of settling/filtering and centrifugation of the algal suspensions, which are necessary in liquid-phase systems to obtain the final dense algal pastes [17], resulting in considerable energy savings.

2. Materials and Methods

2.1. The TL-PBR Structure

The prototype PBRs made in the context of the study are composed of 4 essential parts (Figure 1), namely, the supporting structure; "base" and "cover" for the recirculation and collection of growing media; growth chamber; and peristaltic pump (driving component).

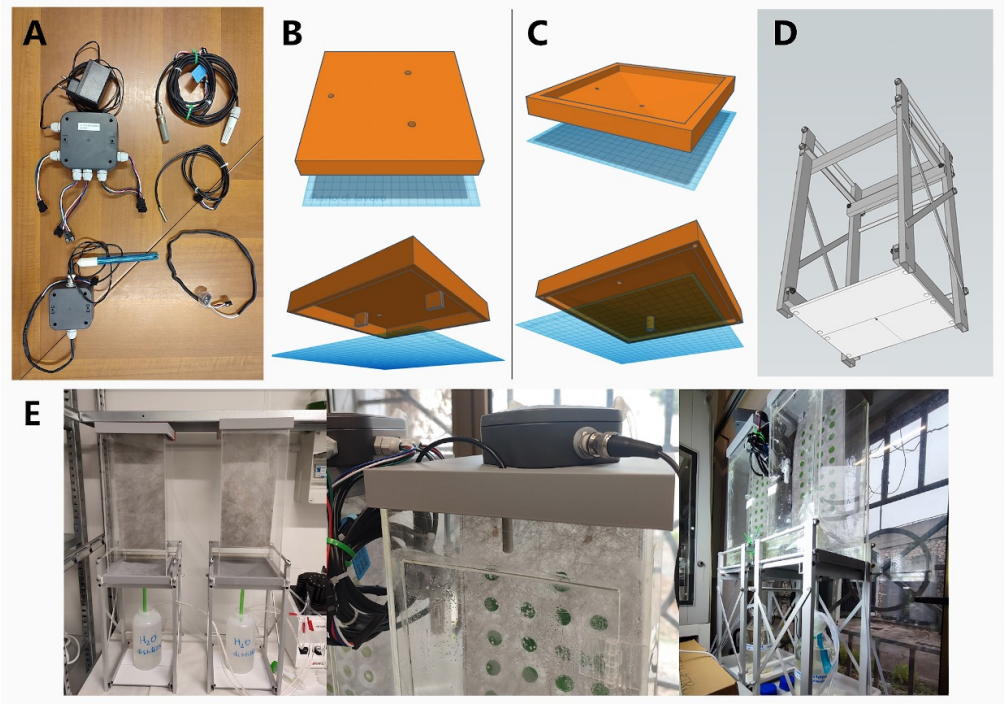

Figure 1. Sensor units of the monitoring system (**A**); renderings of cover and base units, respectively, (**B**,**C**); rendering of the supporting structure (**D**); photos of the TL-PBR prototype cultivation system during testing operations and cultivation tests (**E**).

The supporting structure is designed to hold the base, cover, and growth chamber, and provides vertical stability ensuring a stable centre of mass (Figure 1D). The cover and base (Figure 1B,C) were 3D-designed and printed in ABS plastic polymer. The cover supports the growth panel (source layer) and has apertures for head pipes to distribute the growth medium, while the base features a funnel-shaped structure to facilitate medium recovery and recirculation. Both cover and base include a small off-centre hole for gas exchange.

The growth chamber, made of tempered glass, ensures adequate lighting for the microalgae biofilm (Figure 1E). Inside, the macroporous source layer is securely attached to the cover, with the microporous substrate layer for microalgae growth adhering to it. For these experiments, fibreglass MAT panels with a density of 135 g/m^2 were used as macroporous source layer.

The final essential component of the prototypes is the pump, which functions as the driving mechanism and is the sole element responsible for the electrical energy consumption of the TL-PBR (excluding the monitoring system). For the laboratory tests, a peristaltic pump (model: Perimax 12/6 100–240 V SM 1–80; Spetec GmbH, Erding, Germany) was used. This pump, although having a rated power of 70 W, under real operating conditions and at full load absorbs only 20 W, regardless of the flow rate. These data were measured during the experimental activities using a professional energy meter (model BX11, Trotec GmbH, Verona, Italy). In the conducted tests, the pump was set to ensure a flow rate of 0.5 L/h in each recirculation pipe.

2.2. The Smart Monitoring System

A critical and innovative aspect of this TL-PBR prototype development was the design and implementation of sensor technology for real-time monitoring of environmental process parameters. Specifically, for the cultivation system described in the previous section, a fully customised sensor system was constructed to detect the following parameters in real time: ambient temperature and humidity, growth chambers internal temperature, pH of the culture medium, and light intensity received by the microalgae.

The complete device (Figure 1A) consists of a main unit, the Logger, housed in a 10 × 10 cm Takachi box (Takachi Electronics Enclosure Co., Ltd., Saitama, Japan), which connects to peripheral probes by means of proper junctions. Table 1 provides an overview of the peripherals connected to the Logger.

Table 1. Details of the sensors used for the implementation of the monitoring system.

Parameter	Type of Probe
pH of culture media	Analog pH sensor for acidic solutions Accuracy: ±0.1 at 25 °C
Environmental Light intensity	Photodiode Accuracy: ±0.054 lx
External Temperature and Relative Humidity	Thermo-hygrometer, CMOS-type sensor Temperature accuracy: ±0.2 °C Humidity accuracy: ±2%
Internal Temperature	Programmable Resolution 1-Wire Digital Thermometer Accuracy: ±0.5 °C

The Logger is equipped with a computing unit based on a low-power Wi-Fi/Bluetooth module, which ensures continuous connectivity and data integrity. Regarding the data to be acquired, custom firmware was developed and designed to monitor these parameters at a constant frequency, which can be changed by the user via a configuration page. The system was set to transmit data at the following frequency: every six minutes (the default setting) if there were no significant changes in status; instantaneously if the parameters changed suddenly.

On the web platform connected to the monitoring system (M.E.T. Portal) the user is able to (a) download the data, (b) view it as a raw data table, and (c) visualise its temporal evolution in the form of a graph.

2.3. Cultivation Tests

2.3.1. Experimental Setup

The fundamental process variables that influence the growth performance of microalgal organisms, regardless of strain or cultivation system, include pH of the culture medium, nutrient availability, lighting conditions, and ambient temperature [27]. Taking these factors into account, we conducted 5 cultivation experiments, each lasting 15 days, with the experimental setup summarised in Table 2.

Starting from the conditions of the first experiment (Exp1) (Table 2), each of the next four experiments differed from the previous one in one parameter.

In Exp2, the temperature conditions were modified. Given the thermophilic nature of *G. phlegrea*, its growth was monitored in two different environments: the first at 37 °C (Exp1) and the second at a variable temperature between 28 °C and 35 °C (Exp2), while keeping other variables constant. Specifically, Exp1 was carried out in a closed chamber at a constant temperature of 37 °C, while all other tests were carried out in a laboratory

dedicated to the management of extremophilic strains, equipped with an air conditioning system adjusted to 28 °C. The thermal stability and temporal profile in both conditions were monitored through the sensor system.

Table 2. Summary of the experimental design with the different combinations examined for the operational parameters.

ID Experiment	Duration (Days)	pH	Temperature	Light–Dark Hours	Light Intensity (Lux)	Experimental Control	Test	Fresh Feeding Frequency of Culture medium
Exp 1	15	2.5	37 °C	16:8	~1000	Allen medium (1×)	Wastewater	Never (batch)
Exp 2	15	2.5	28–35 °C	16:8	~1000	Allen medium (1×)	Wastewater	Never (batch)
Exp 3	15	2.5	28–35 °C	16:8	~32,000	Allen medium (1×)	Wastewater	Never (batch)
Exp 4	15	2.5	28–35 °C	16:8	~32,000	Allen medium (1×)	Wastewater	Every 2–3 days (semi-batch)
Exp 5	15	2.5	28–35 °C	16:8	~32,000	Allen medium (1×)	Wastewater	Continuously

In Exp3, while lighting was provided according to 16:8 h light–dark cycles (regulated by timers) in all of the tests, the intensity of the light beam during daylight hours was modified. In particular, Exp1 and Exp2 were performed using only the ceiling lighting (fluorescent lamps) available in the two environments, which could provide approximately 1000 lux. In the other experiments, artificial lighting was provided by a 6000 K LED disc positioned at a distance of about 30 cm from the growth panels, resulting in about 32,000 lux, which corresponds to real sunlight at a low intensity.

In Exp4 and Exp5, the frequency of medium renewal was increased. In the initial three tests (Exp1, Exp2, Exp3), 1 L of each culture medium was provided on day 0 and continuously recirculated by the peristaltic pump over the 15-day test period. In contrast, for Exp4, each 1 L bottle of medium was replaced with fresh medium every 2–3 days, coinciding with sample collection. Furthermore, in Exp5, an attempt was made to simulate a continuous supply of fresh medium by configuring the PVC pipes of the TL-PBR to draw fresh medium from 50 L tanks and pour it into different tanks. These tanks were changed as required, typically every 2 days, to ensure an uninterrupted flow.

2.3.2. Culture Media Preparation

For the 5 experiments, according to [15], the following culture media were used: (i) primary urban wastewater (W) as test; (ii) Allen 1× culture medium (A) under autotrophic conditions as experimental control.

The urban effluents were collected from a CAS (Conventional Activated Sludge) treatment plant located in the city of Eboli (Province of Salerno, Campania Region, Italy), at the inlet of the activated sludge oxidation tanks, hereby referred to as primary effluents. The sampling point was chosen because, at this stage of the plant design, the effluents have only undergone pre-treatment to remove grease, sand, and coarse material. Therefore, they still contain all the dissolved organic and inorganic pollutants that need to be converted and removed by oxidative treatments, so they are extremely valuable as pool of macro- and micro-nutrients from which micro-algae can derive food for their growth [15]. The composition of urban wastewater can vary over the seasons due to various environmental or anthropogenic factors, such as occasional tourism, meteorological events or seasonal agricultural and zootechnical practices [24]. Therefore, in order to ensure the use of

homogeneous effluents in all experiments, a sufficient volume of effluents was collected and prepared during the sampling campaign to cover all experiments.

Briefly, after sampling, the effluents were subject to microfiltration with high-speed cellulose filter paper (model: 9.045 807, LLG-Labware GmbH, Meckenheim, Germany), followed by pH adjustment to 2.5 by adding H_2SO_4 and using an electronic pH meter calibrated with buffer solutions (model: 6.263 691, LLG-Labware GmbH, Meckenheim, Germany). The acidified effluents obtained from this step were then sterilised in an autoclave at 121 °C for 30 min, then stored at 4 °C until use.

For the Allen medium, it was prepared according to the protocol of the reference microalgal collection for the strain used (published and available on the website https://www.acuf.net/wp-content/uploads/2025/01/1.pdf—accessed on 23 May 2024) and adjusted to pH 2.5 by adding H_2SO_4 and using a pH meter. The same sterilisation and culture medium storage procedures were then applied, i.e., autoclaving at 121 °C for 30 min followed by storage at 4 °C. As mentioned at the beginning of this section, the control used here did not include the addition of organic carbon sources, allowing the biomass to grow solely by autotrophic metabolism.

As a final note, since Exp5 was designed to simulate a continuous supply of fresh culture medium, this required the use of large volumes of medium to reach the end of the 15 days. In particular, for a continuous flow of 0.5 L/h in each recirculation tube and 2 tubes for each growth chamber, it was necessary to prepare at least 360 L of both W and A for Exp5 alone.

2.3.3. Initial Biomass Inoculum Preparation and Growth Monitoring

The strain selected for the experiments (*G. phlegrea* ACUF 784.3, Figure 2) was obtained from the Algal Collection of the University of Naples Federico II (www.acuf.net, accessed on 6 May 2024). The algal stock was prepared by isolating the strain through streaking on agar plates. The colonies were then inoculated in liquid Allen medium (pH 2.5) and incubated at 37 °C under continuous fluorescent illumination (45 µmol photons m^{-2} s^{-1}), with weekly medium refreshment.

At the beginning of each growth test, an appropriate volume of algal stock was collected and centrifuged at 4000 rpm for 5 min at room temperature. The sedimented biomass was washed twice with ultrapure water and finally suspended in a 30 mL conical tube, resulting in a highly concentrated algal suspension. To calibrate the concentration of the algal suspension for the start of the experiments, three test samples were prepared from this volume. Specifically, for each test sample, a 500 µL aliquot was taken from the 30 mL suspension and then inoculated onto a 0.4 µm mesh hydrophilic polycarbonate filter (NucleporeTM Track-Etch Membrane—WhatmanTM, Maidstone, UK), which served as the substrate layer. Custom-made cylindrical shapes were used to ensure a well-centred and defined spatial distribution of the biomass on the filter, with cellulose filter paper used to absorb excess supernatant filtered through the microporous membrane. The final result of the preparation was a polycarbonate microporous disc with a dense algal biomass well attached, an example of which is shown in Figure 2B. The three test discs prepared using this procedure were placed in an oven at 65 °C for 30 min to dry the biomass. After this time and knowing the weight of each disc (standardised and certified by the manufacturer), the net weight of the dry biomass alone from these discs was determined gravimetrically, thus allowing us to calculate the corresponding average surface distribution of the biomass on the substrate layer, expressed as grams per square metre. This allowed the calculation of the appropriate dilution factor to be applied to the initial 30 mL of concentrated suspension to achieve the desired value for the start of the tests, namely an average surface distribution of approximately 6 g/m^2.

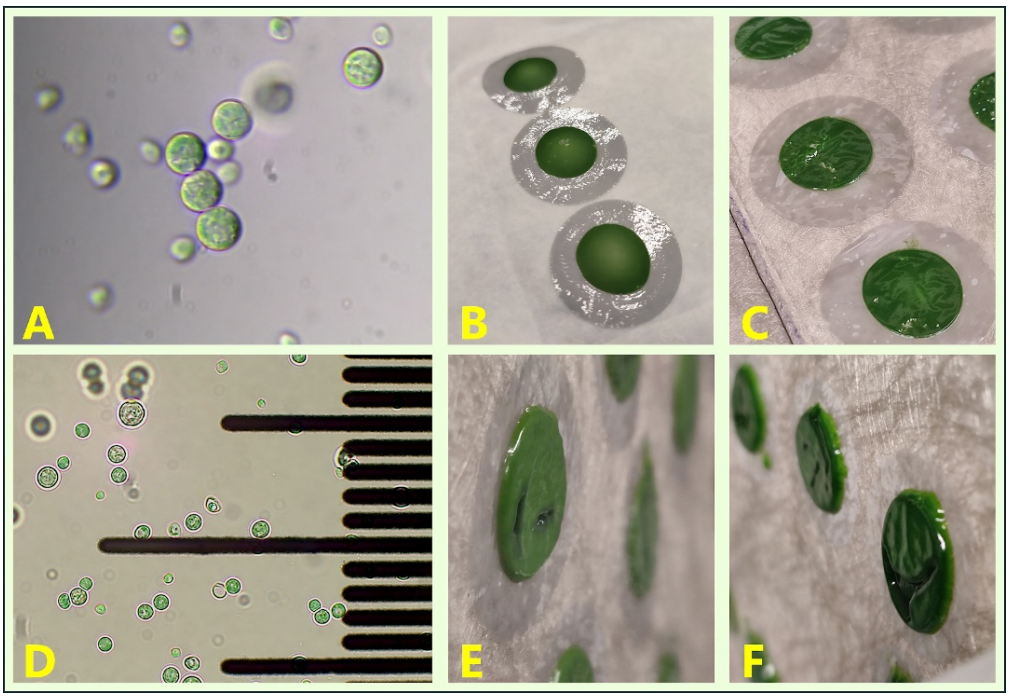

Figure 2. *G. phlegrea* ACUF 784.3: personal shot captured using an optical microscope at 100× magnification (**A**); test discs usually prepared for the start of cultivation experiments (**B**); discs of biomass on polycarbonate micromembranes (substrate layer), adhered to the fibreglass panel (source layer), and ready for the start of cultivation experiments (**C**); personal shot captured using an optical microscope with 40× magnification and 10 µm sensitivity graduated slide (**D**); an example of dense algal biofilm developed during some preliminary TL-PBR trials (**E**,**F**).

With the algal suspension diluted to the appropriate concentration, all the discs required for the tests were finally prepared using the same procedures as for the three test discs. At the start of every experiment, a sufficient number of discs were prepared to carry out the following analyses: biomass growth monitoring; EA-IRMS analysis of biomass (in Exp3); SEM-EDS observations and microanalysis on the biomass (in Exp3). The frequency of sampling in all experiments was every 2–3 days from day 0 to day 14, for a total of 15 days.

2.4. Biomass Characterisation

In addition to the gravimetric determination of biomass growth in all experiments, further compositional and morphological characterisation of the biomass was carried out. Specifically, EA-IRMS and SEM-EDS analyses were performed on samples collected during Exp3. Exp3 was chosen as a representative experimental sample for these characterisations because it not only simulated environmental conditions (light and temperature) that could easily be achieved in a full-scale cultivation environment, but it also allowed us to observe the physiological response of the microalgae under management conditions where media renewal could be scheduled for longer periods (at least after 2 weeks).

2.4.1. EA-IRMS Analysis

Due to differences in mass number, isotopes of the same element exhibit different physical properties, leading to fluctuations in their relative abundances in nature through chemical, physical or biological processes (fractionation phenomena). For example, processes such as metabolic activity in organisms can lead to enrichment or depletion of specific isotopes, a mechanism that is also influenced by the specific isotopic signal of the matrices with which they come into contact [28]. The isotopic composition of elements in

environmental matrices is typically expressed in delta notation (δ), which indicates the deviation in parts per thousand (‰) of the isotopic ratio of interest relative to that of a reference material of known isotopic composition [29]. A δ value greater than 0 indicates that the analysed matrix has a higher content of heavier isotopes compared to the standard. From another perspective, by analysing the isotopic signal of a given matrix over time and observing how it deviates from its original signal (whether it increases or decreases), information about ongoing interactions with surrounding matrices can be obtained.

Here, the application of EA-IRMS aims to quantify carbon and nitrogen isotopic ratios and concentrations in the biomass, assessing temporal variations and identifying interaction pathways with the W and A matrices in contact with the microalgae, as reported by [15]. To achieve this, it was also necessary to characterise the initial isotopic signature of W and A, in addition to the EA-IRMS analysis of the algal biomass over time.

To prepare the biomass for EA-IRMS measurements, a sample disc removed from the reactor was placed in an Eppendorf tube containing 2 mL of distilled water, exposed to an ultrasonic bath for 5 min, and finally vortexed for a few seconds to recover all the biomass from the disc. The tubes were then centrifuged at 10,000 rpm for 10 min, after which the supernatant was removed, and the biomass was frozen at −80 °C and lyophilised without further pre-treatment.

To obtain the solid matrices necessary to characterise the initial isotopic signal of C and N in the W and A culture media, the following procedure was followed. A volume of 100 mL of acidified and sterilised medium was frozen at −80 °C without further treatment and lyophilised, thus obtaining residual solid material ready for analysis.

To carry out the analyses on the solid matrices thus obtained, a quantity of each sample was weighed in triplicate in the following ranges using a high sensitivity analytical balance (AT20, Mettler Toledo, Milano, Italy, ±2 μg): biomass in the range 0.4–0.8 mg, solid residue of A in the range 1.5–2.0 mg, solid residue of W in the range 3.0–5.0 mg. The weighed material was encapsulated in 4 × 6 mm tin capsules (Tin capsules for solids, Santis Analytical Italia, Gerenzano, Varese, Italy). These samples were then analysed using an integrated system consisting of an IRMS apparatus (Delta V Advantage-Thermo Fisher, Waltham, MA, USA) coupled to an Elemental Analyzer (1112 Series-Thermo Fisher, Waltham, MA, USA) for the simultaneous measurement of total C and N (%) and their relative isotopic ratios $\delta^{13}C$ and $\delta^{15}N$ (‰). The reference standards are Vienna PDB for the $\delta^{13}C$ ratio ($=^{13}C/^{12}C$) and air for the $\delta^{15}N$ ratio ($=^{15}N/^{14}N$).

2.4.2. SEM-EDS Analysis

In addition to the EA-IRMS, it was decided to include a morphological characterisation of the algal biofilm adhering to the discs with a double objective. Firstly, to determine whether there were any significant morphological differences observed over the cultivation days as well as between the biomass grown with urban wastewater and that grown with the standard culture medium. Secondly, microscopic observations were necessary to check for traces of possible contamination by competing organisms, identified by the possible presence of fungal hyphae or organisms morphologically distinct from the algal population. To this end, a part of the collected samples was assigned for SEM-EDS analysis.

In order to prepare the samples for analysis on each sampling day, aiming to preserve as much as possible the stratification acquired by the biofilm during its growth, the discs were laid flat on Petri dishes and then fixed in place with small adhesive strips. They were then frozen at −80 °C and lyophilised.

Finally, the biomass discs were gently detached from the polycarbonate membranes and mounted on aluminium specimen stubs with double-sided tape and coated with a layer of gold with a Q150R ES Sputter Coater (Quorum Technologies, Lewes, UK) and observed

via Scanning Electron Microscopy (SEM) using a Zeiss EVO 15 HD VPSEM (Oberkochen, Germany) operating at 12 kV accelerating voltage to record images, coupled with an Oxford Instruments Microanalysis Unit (Abingdon, UK) with Xmax 80 EDS detector for microanalyses. Standard details used for EDS calibrations are reported in [30].

2.5. Analysis of NH_4^+, NO_3^- and PO_4^{3-} Content in W e A

In addition to biomass samples for EA-IRMS and SEM-EDS analyses, samples of the culture media from Exp3 were collected over time to investigate how the levels of specific ionic species in the growth media varied over the course of 15 days, in particular ammonium, nitrate and phosphate. For this purpose, 50 mL of supernatant was collected every 2–3 days and analysed using a portable spectrophotometer (model: DR1900, Hach Lange S.r.l., Milan, Italy) and the appropriate kits from the same manufacturer, certified to perform colorimetric tests in cuvettes. Specifically, the Hach LCK303 and LCK304 kits, based on the indophenol blue method, were used for ammonium; the Hach LCK339 kit, based on the 4-nitro-2,6-dimethylphenol reaction product, was used for nitrate; and the Hach LCK348 and LCK350 kits, based on the phosphomolybdate blue method, were used for phosphate. Trial analyses were carried out before the tests to determine the dilution factor to be applied to the samples in order to fall within the measuring ranges of the kits.

2.6. Data Processing and Statistical Analysis

For biomass growth monitoring, discs were prepared in 3 biological replicates for each condition A and W and each was weighed 3 times (n = 9). For EA-IRMS analyses, 3 biological samples were taken from each disc to account for any biomass heterogeneity (n = 3). For contaminant measurements, chemical analyses were performed in triplicate on each liquid sample, and each vial containing the reacted sample was spectrophotometrically observed twice to account for any homogeneity in sample mixing (n = 6).

Based on our experimental growth data, although this study was not primarily focused on identifying the most accurate mathematical model to describe the metabolic processes of *G. phlegrea*, we nonetheless explored a possible approximation of biofilm growth dynamics over extended time periods. To this end, we applied simple regression models that do not incorporate specific environmental growth variables. We tested and compared three approaches: quadratic polynomial regression, three-parameter logistic regression, and two-parameter exponential regression.

In these models, the response variable y represents surface biomass density, while the predictor variable x corresponds to time. The reference equations for the three regression models are as follows:

- Second-degree polynomial, $y = ax^2 + bx + c$. In this equation, a and b are the growth rate coefficients of the second and first-degree functions of x, respectively, while c represents the value of the surface density at time 0;
- Three-parameter logistic, $y = c/\{1 + exp[-a(x - b)]\}$. In this equation, a represents the growth rate, b is the value of x for which $y = c/2$ and c represents the asymptotic algal density value reached at saturation according to this model;
- Two-parameter exponential, $y = a\ exp(bx)$. In this equation, a represents the surface algal density at time 0 and b represents the growth rate.

The statistical analyses and the calculation of the parameters a-b-c in the 3 models were performed through the use of the software JMP (version 17 Pro, SAS Analytics).

The following statistical parameters were considered to determine the fit and significance of the evaluated models: Akaike Information Criterion corrected (AICc), Bayesian Information Criterion (BIC), Sum of Squared Errors (SSE), Mean Squared Error (MSE), Root Mean Squared Error (RMSE) and the R-squared coefficient (R^2). For these parameters,

unlike the R^2 coefficient, the mathematical model that best describes a given time series is the one that gives the lowest values within a group of models; the greater the numerical difference between one model and another, the better it describes the phenomenon.

The significance level used in all statistical analyses is $\alpha = 0.05$. Data curation, evaluation of statistical parameters and graphs were performed using JMP17 Pro (SAS Analytics) and Microsoft Office Excel (version 2403).

3. Results and Discussion

3.1. Metabolic Response and Connections with Isotopic, Spectrophotometric, and Monitoring Data

With the experimental setup investigated in this study, it was possible to compare the biomass growth response across the five environmental conditions shown in Table 2. Figure 3 shows, for W and A, respectively, the biomass growth trends in terms of surface density (expressed as grams of dry algal biofilm per unit area of cultivation) in the five tests.

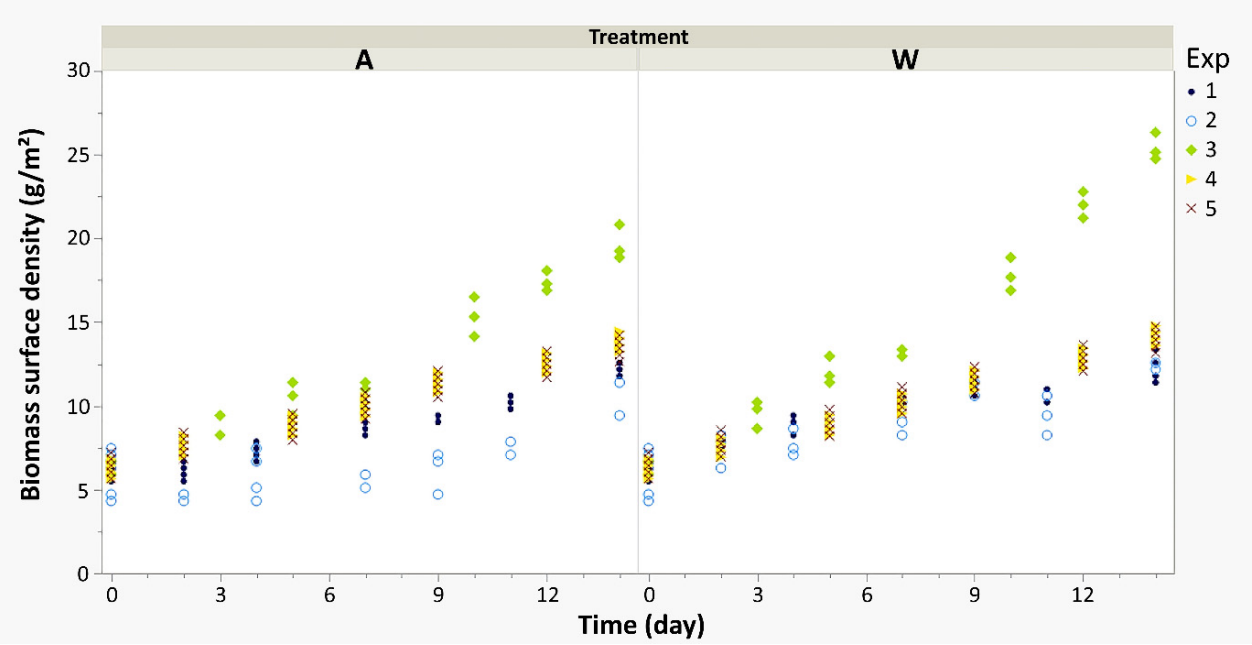

Figure 3. Time trend of biomass grown with culture medium A (Allen medium) and W (wastewater), as surface density.

Upon initial observation, the operational conditions set in Exp3 proved to be the most effective for both W and A, resulting in significantly higher growth at the end of the 15-day observation period. Specifically, with W, a final average surface density of biomass of 25.4 g/m^2 was recorded, which is a +91% increase compared to the average value of 13.3 g/m^2 obtained from the other four experiments (Figure 3). With the A culture medium, Exp3 recorded a surface density 56% higher than the average obtained in the other four tests (19.6 vs. 12.6 g/m^2).

With regard to the experimental conditions that were explored in the five experiments, it appears that temperature variations within the range investigated in Exp1 and Exp2 did not result in substantial differences in biomass growth (Figure 3). It seems that the transition from Exp2 conditions to those of Exp3, with the introduction of a higher intensity light source, proved to be a successful strategy, leading to the previously mentioned final yields. In the subsequent two tests (Exp4 and Exp5), no substantial differences were observed between the semi-batch mode of Exp4 and the continuous mode of Exp5. However, it seems that the choice to provide fresh medium with progressively higher frequency than

the batch mode of Exp3 was counterproductive, leading to less satisfactory final yields (Figure 3). One possible explanation for this result is that, within this cultivation system and given the nutrient bioavailability in the growth media, the algal community might have adopted a metabolic strategy that meets its needs without requiring immediate access to fresh nutrients. Specifically, the microalgal community in this system might require an initial period to adapt to the growing conditions, during which it might establish a dynamic chemical equilibrium with the culture medium, and then progressively exploit all available resources [31]. Varshney et al. [32] indicated that microalgae require a certain acclimation period when exposed to environments with higher CO_2 concentrations than those to which they are used. In this context, the continuous addition of fresh culture medium—potentially containing increased levels of dissolved CO_2—may have triggered a transient adaptation phase, temporarily influencing cellular metabolism and growth dynamics. This phenomenon could explain the observed interference, as sudden fluctuations in CO_2 availability may affect photosynthetic efficiency, carbon assimilation rates, and intracellular pH homeostasis, all of which play a crucial role in microalgal adaptation to environmental changes.

Compelling evidence supporting this interpretation emerged from EA-IRMS and spectrophotometric investigations. The first revealed the presence of interaction mechanisms with matrices external to the biomass; on the other hand, the second seemed to suggest a temporary equilibrium in the concentration of ionic species in solution.

Analysing the results of the two methodologies in detail and starting with the EA-IRMS analysis, Table 3 provides a summary of the results obtained, including the elemental composition of carbon and nitrogen (%) and the isotopic signals of the analysed matrices.

Table 3. Results of EA-IRMS analysis, i.e., measurements of stable isotope ratios $\delta^{13}C$ and $\delta^{15}N$, and C and N content of the analysed matrices. Data are expressed in aggregate form and reported as mean ± standard deviation (SD) versus their standards, whereas VPDB is the Vienna Pee Dee Belemnite. Allen medium and wastewater represent the initial compositional signals of each of the two culture media, respectively.

	$\delta^{13}C$ (‰) vs. VPDB	**$\delta^{15}N$ (‰) vs. Air**	**% C**	**% N**
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Biomass grown in A	−26.0 ± 0.4	−13.6 ± 0.5	48.5 ± 1.7	9.3 ± 0.8
Biomass grown in W	−26.0 ± 0.5	−11.0 ± 1.6	48.3 ± 1.6	9.2 ± 0.8
Allen medium (dry residue)	-	−0.9 ± 0.2	-	21.5 ± 0.3
Wastewater (dry residue)	−26.3 ± 1.8	5.1 ± 1.4	0.9 ± 0.1	1.3 ± 0.1

From a content perspective, the biomass showed an average C and N concentration of 48.4% ± 1.6% and 9.2% ± 0.8%, respectively, with no significant differences observed between biomass grown in contact with W and that grown in contact with A. The numerical result appears to be consistent with the average values reported in the relevant literature for species of this algal family [33] and is slightly higher than the values reported by [15] for the same strain. Observing the temporal trends of %C and %N for both W and A (Figure 4), it appears that they proceed in a similar way. It is interesting to note that while the concentration of C remains relatively stable over the 15-day observation period ($R^2 < 0.1$), there is a percentage accumulation of N over time (from 8% to 10%, $R^2 > 0.6$), which might be explained by an intracellular accumulation of nitrogen-containing macromolecules. When we consider the environmental conditions of Exp3 during which the samples were collected (Table 2), it seems reasonable to hypothesise that microalgae exposed to a light intensity of approximately 32,000 lux may have undergone increased production over time of photosynthetic proteins, such as phycobilins. This hypothe-

sis is supported by several studies demonstrating a positive relationship between light intensity received by microalgal cultures—including those of the Galdieriaceae family—and their phycocyanin content [16,34]. In general, most microalgae, including species of the genus *Galdieria*, are typically cultivated under light intensities ranging from approximately 2000 to 4000 lux (corresponding to ≈30–60 µmol photons·m^{-2}·s^{-1}) [11,20]. However, increasing the light intensity to values between 5000 and 6000 lux (approximately 100 µmol photons·m^{-2}·s^{-1}) has been shown to enhance pigment production [35–37]. Although higher intensities such as those tested in the present study (32,000 lux) are not commonly adopted, Wang et al. 2020 [34] demonstrated that light intensities approaching 600 µmol photons·m^{-2}·s^{-1} (≈32,000–36,000 lux) effectively amplified the photoinductive response, resulting in increased synthesis of photosynthetic pigments.

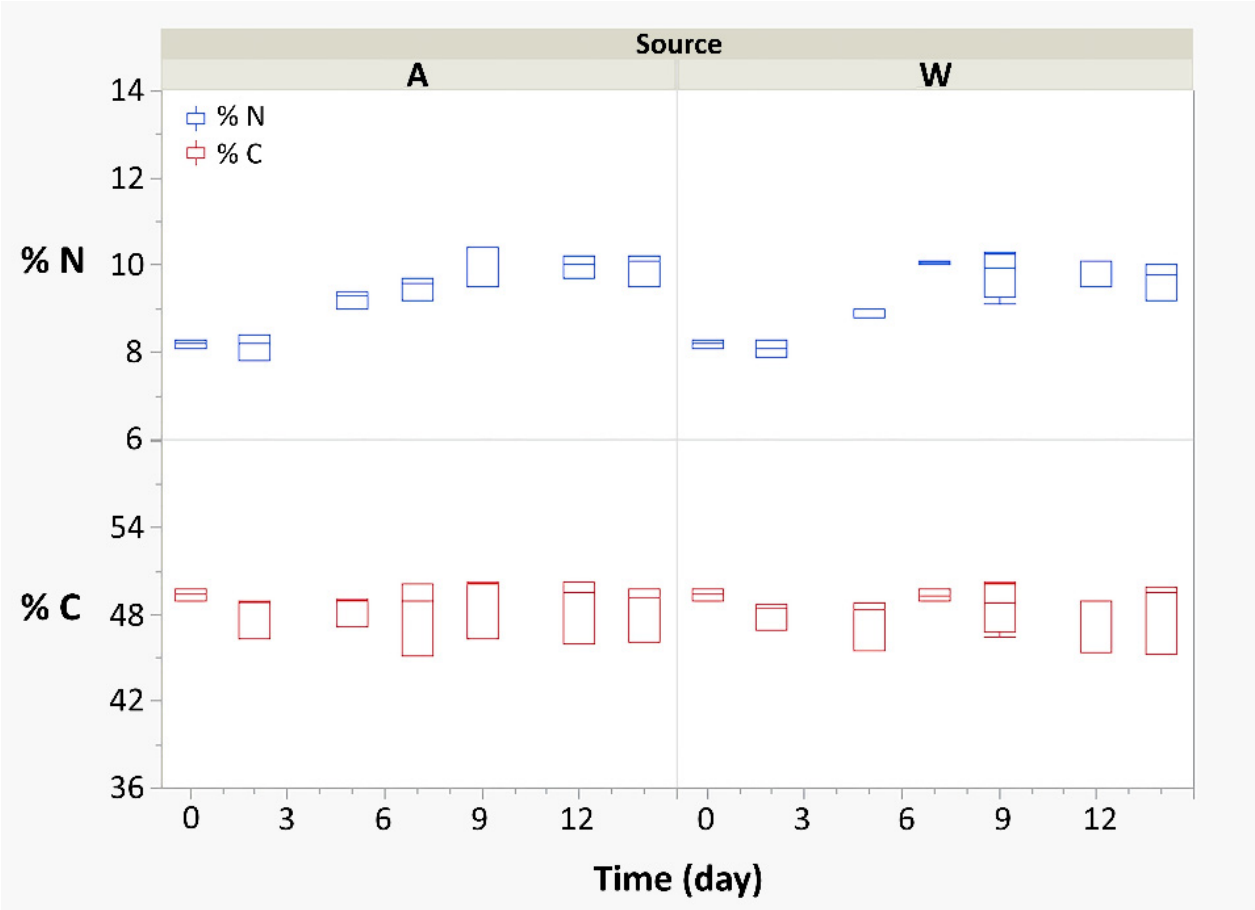

Figure 4. Time trend of C and N concentration in biomass grown with A (Allen medium) and W (wastewater) sources.

Concerning the information derived from isotopic data, as mentioned in Section 2.4.1, whenever a system exchanges with external matrices, it is inevitably influenced by the isotopic signature of that matrix according to appropriate mixing models [28]. In this case, despite the two different growth media, the $\delta^{13}C$ signal (Figure 5) showed a slight shift towards higher values for both A (R^2 = 0.6) and W (R^2 = 0.7). This suggests a common carbon-related metabolic pathway based on a source characterised by a higher $\delta^{13}C$ signal. This source could be atmospheric air (for photosynthesis), which typically has $\delta^{13}C$ values around −8‰ [38] and can circulate freely in both growth chambers through the holes in the base and cover of the PBR. This result suggests the predominance of photosynthetic processes in both growth conditions, as evidenced by the average $\delta^{13}C$ value of approximately −26‰ (Table 3) observed throughout the study period, which is

typical of terrestrial plants with a C3 metabolism [39]. In line with these findings, ref. [40] confirmed the presence of photosynthetic pathways in microalgal biomass similar to those in C3 terrestrial plants.

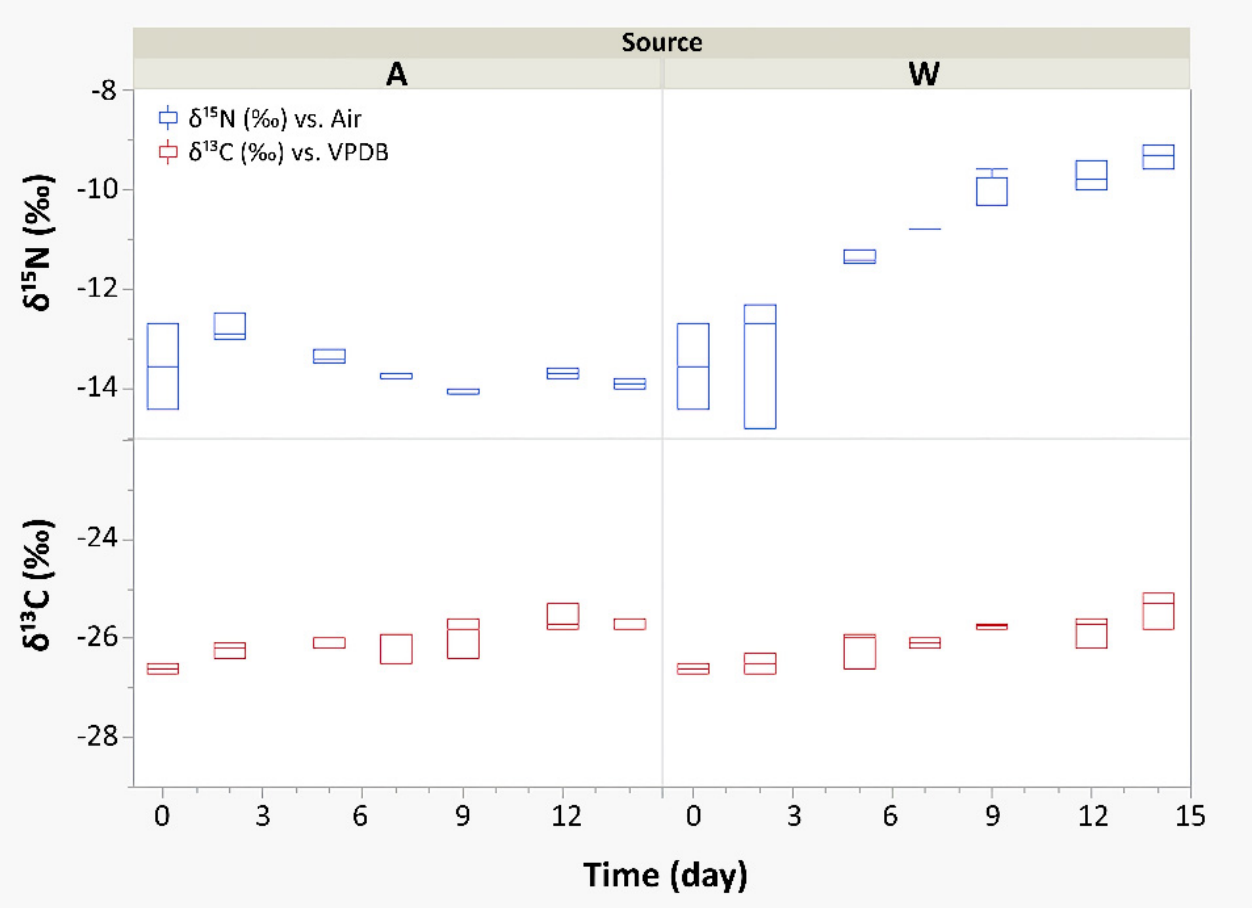

Figure 5. Time trend of $\delta^{13}C$ and $\delta^{15}N$ isotope ratios in biomass grown with A (Allen medium) and W (wastewater) sources.

Regarding nitrogen, the assimilation mechanisms employed by microalgae rely on the transmembrane transport of inorganic nitrogen from the aquatic environment [41]. Consequently, while no significant alterations of isotopic signal ($R^2 = 0.3$) were observed in the A standard (as expected), the increase in $\delta^{15}N$ values observed in W ($R^2 = 0.8$) (Figure 5) correlates with the increase in %N observed in Figure 4, indicating a clear uptake of nitrogen by microalgae from the wastewater source, the latter being characterised by a $\delta^{15}N$ signal much higher than that of the initial biomass (Table 3). It is also worth noting that the $\delta^{15}N$ signal observed in the wastewater medium is consistent with the presence of fertilisers and organic nitrogen [42], which accurately reflects the fact that the wastewater treatment plant supplying the effluents used in this study is situated in a rural area with extensive agricultural and livestock activities.

The data obtained from spectrophotometric analyses of ammonium, phosphate and nitrate content in the culture media add interesting insights to the results of EA-IRMS analyses. When examining the behaviour of these ionic species in culture medium W, no significant trend was observed over the days of observation (Figure 6). Instead, their concentrations fluctuated around average values of 32.5 ± 2.8 mg/L for NH_4^+, 2.3 ± 0.5 mg/L for PO_4^{3-}, and 0.6 ± 0.1 mg/L for NO_3^-. Similar fluctuations were also observed in the standard culture medium A (data available in the Supplementary Materials).

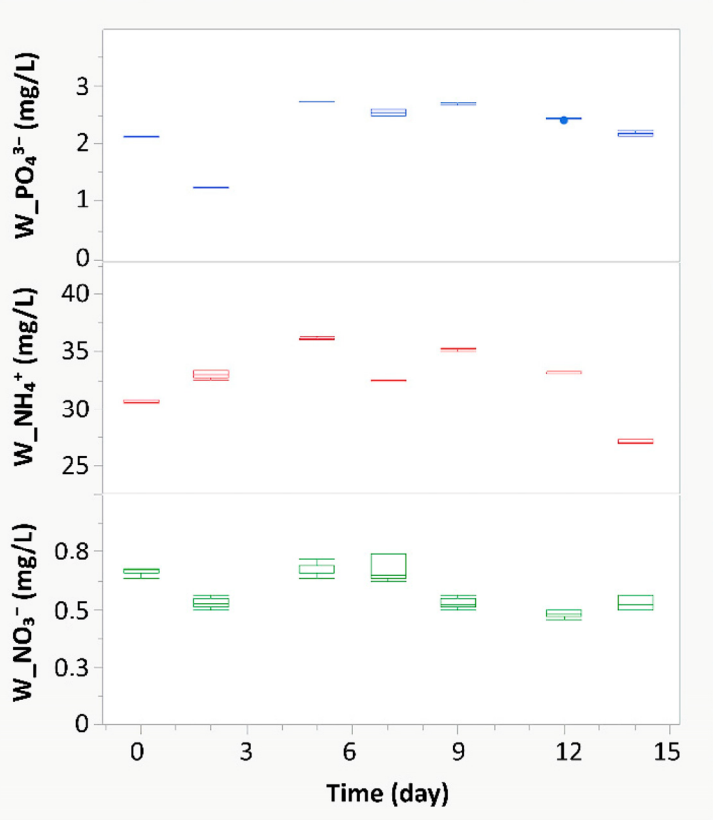

Figure 6. Time trend of NH_4^+, NO_3^- and PO_4^{3-} in culture medium W during Exp3.

This trend, observed in both A and W, may indicate the presence of complex physicochemical and biological mechanisms regulating nutrient availability within the culture system. Since in Exp3 the culture medium was continuously recirculated and not replenished throughout the entire observation period, these fluctuations cannot be attributed to external nutrient inputs. Instead, the data suggest an internal dynamic equilibrium where multiple factors contribute to the stabilisation of ionic species concentrations.

The pattern observed in this study aligns with existing knowledge on microalgal nutrient metabolism. Various studies have demonstrated that the assimilation and release of nitrogen and phosphorus in microalgae are strongly influenced by environmental parameters such as pH, temperature, light exposure, and the ionic balance within the culture medium [43]. When intracellular concentrations of NH_4^+ and NO_2^- surpass the cells' capacity for assimilation, microalgae may expel these ions into the surrounding medium as a detoxification response to avoid nitrogen stress [44].

While ammonium is generally the preferred nitrogen source for microalgal growth, many species including *Galdieria* spp. can also utilise organic nitrogen compounds, particularly urea, amino acids, and small peptides, which are commonly present in wastewater [43,45–47]. Given that the metabolic pathways of carbon, nitrogen, and phosphorus are interdependent, variations in the availability of one of these nutrients can significantly impact the uptake and transformation dynamics of the others, ultimately shaping the nutrient equilibrium within the culture system [43].

The literature further supports that microalgae of the genus *Galdieria*, like many other species, regulate NH_4^+ and NO_3^- uptake in response to external conditions. Ammonium metabolism produces protons (H^+) as a by-product, leading to a local decrease in pH, whereas nitrate assimilation increases pH by consuming protons [41]. The interplay between these processes contributes to pH stability in the culture system, depending on the balance of nitrogen sources available in the medium.

Here, the implementation of a sensor system on the TL-PBR provides a significant support in the analysis of these data and to better understand these interactions. The results (Figure 7) show that pH levels oscillated around the initial value, maintaining a relatively stable pattern throughout the experimental period. This behaviour suggests that the culture system was able to maintain a chemical equilibrium over time, supporting the hypothesis of self-regulating metabolic processes that counteract excessive fluctuations in nutrient concentrations.

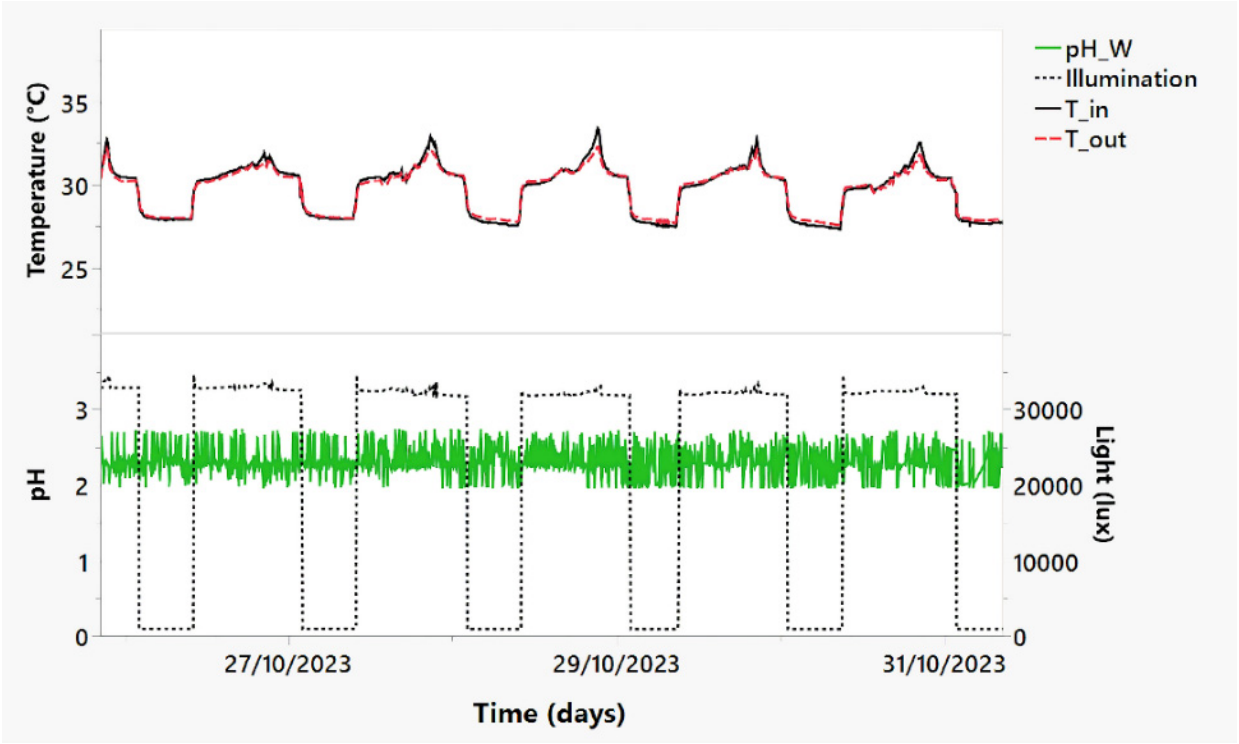

Figure 7. Real-time trend, during Exp3, of the parameters pH, illumination, and temperature inside and outside the growth chambers.

Moreover, Figure 7 also provides valuable insight into environmental parameter variations during the experiment. In particular, it highlights the direct influence of LED lighting on temperature dynamics, showing that pH stability was maintained despite daily fluctuations in ambient temperature.

Overall, these findings suggest that the lack of significant nutrient depletion or accumulation in the medium does not indicate an absence of metabolic activity but rather the presence of compensatory physiological and chemical mechanisms that dynamically regulate nutrient levels within the culture system.

3.2. SEM Characterisation and Projections of Biomass Yields Obtainable from the System

In addition to the metabolic investigations, two important focuses of this study were (i) the potential for reusing biomass in biotechnological applications and (ii) the long-term biomass yields.

Regarding the first aspect, as suggested in Section Rationale Behind the Selection of Twin-Layer Cultivation Technology, the application potential increases with the level of purity of the harvested biomass. Here, the TL-PBR used in this study allows the production of homogeneous and uncontaminated biomass within the observation period. SEM analyses of the biomass (Figure 8) showed that the algal biofilm had a very homogeneous structure, free from foreign organisms/bodies, with no significant morphological differences observed either over time (day 2 vs. day 14) or relative to the culture medium from which

the microalgae derived their nutrients. Any irregularities within the algal biofilm depicted in Figure 8 are not due to the medium used, but to the drying phase of the biomass discs via lyophilisation, which can cause cracks as a result of the mechanical stress associated with the operating pressure [48]. Examining the cellular aggregates, it becomes apparent that cells seem interconnected by an external matrix (Figure 8). This morphological trait likely arises from the cells' capacity to secrete extracellular polymeric substances (EPSs) rich in polysaccharides [49], as confirmed by the results of the EDS investigations. Compositional analyses via EDS of the algal biofilm surface detected primarily carbon and oxygen, alongside traces of other elements naturally present in the culture media employed (for example sulphur, phosphorus, potassium, calcium, magnesium, and others). Complete EDS spectra of the analysed samples are provided in the Supplementary Materials. While the SEM characterisation in this study focused on the horizontal plane to examine the surface distribution of cells, future experimental investigations will focus on a more detailed study of the vertical section of the algal biofilm. This approach will provide more information on the porosity and morphology of the cells within the different photosynthetic layers, thus allowing further clarification of the structure and functions exhibited by the algal community.

Regarding the second key objective of this study, namely the long-term biomass yields achievable by the system, this aspect was explored through statistical extrapolation rather than mechanistic modelling. Specifically, we applied simple predictive regression models to the temporal trends observed in the five experiments, as a means of statistical approximation. Table 4 presents the statistical parameters obtained for the three regression models tested—quadratic polynomial, three-parameter logistic, and two-parameter exponential—for the two conditions, A and W, as detailed in Section 2.6. The full statistical report of the models applied to the growth data is available in the Supplementary Materials.

Table 4. Comparison of the three mathematical regression curves fitted to the growth data for conditions A and W, respectively.

Treatment	Model	AICc	BIC	SSE	MSE	RMSE	R^2
A	Second-degree polynomial	678.35	736.57	142.71	0.47	0.69	0.96
	3-parameter logistic	773.64	831.85	193.12	0.64	0.80	0.95
	2-parameter exponential	787.01	827.41	208.62	0.68	0.83	0.95
W	Second-degree polynomial	716.85	775.07	161.26	0.54	0.73	0.97
	3-parameter logistic	718.05	776.26	161.87	0.54	0.73	0.97
	2-parameter exponential	763.52	803.93	193.63	0.63	0.80	0.96

Although the quadratic polynomial regression exhibited the best statistical fit (Table 4), all three models produced similar performance metrics, suggesting high statistical reliability within the 15-day observation period. However, these models were applied purely as statistical tools for interpolation and extrapolation, rather than as biologically representative frameworks. As such, they do not capture the metabolic or physiological mechanisms governing the growth of *G. phlegrea*. Future studies aiming to better describe the system's long-term behaviour should consider incorporating biologically informed models that account for environmental variables and resource limitations.

When analysing the long-term projections (Supplementary Materials), the models consistently identified Exp3 as the most favourable operating condition, with higher biomass accumulation observed in wastewater (W) compared to the standard culture medium (A), as illustrated in Figure 9. This finding aligns with previous literature [13,14].

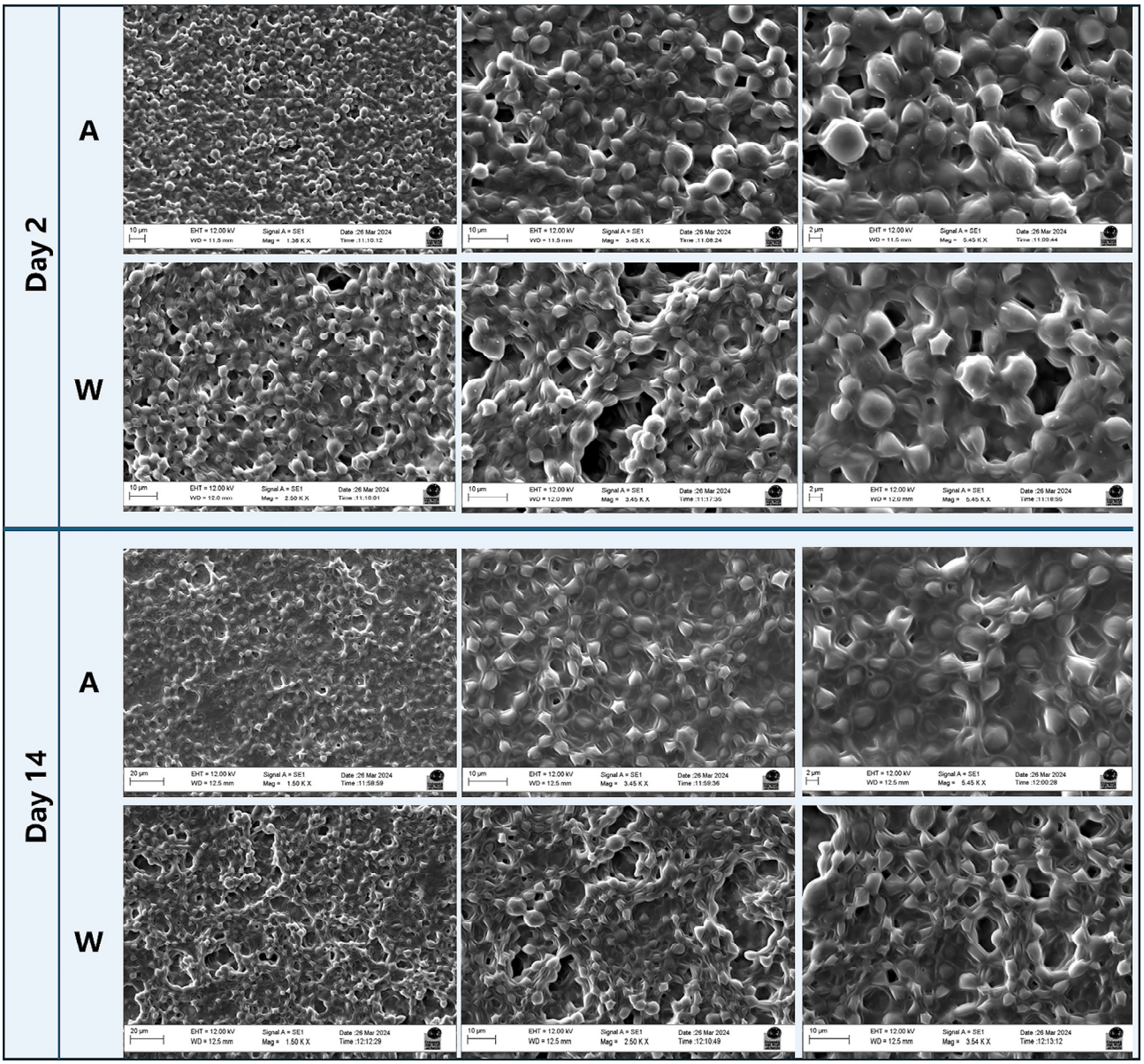

Figure 8. SEM observations on biomass samples grown with W and A and collected at days 2 and 14.

For Exp3, the extrapolated growth trends in Figure 9 suggest potentially significant long-term biomass yields. Among the models tested, the exponential regression provided the most optimistic projection, indicating that a surface biomass density of 1 kg_{DW}/m^2 could be achieved within approximately two months (54 days for W, 64 days for A).

It is important to emphasise that these projections are purely theoretical. Both polynomial and exponential models lack saturation constraints, mathematically suggesting continuous biomass accumulation. However, in real-world conditions, multiple limiting factors—including nutrient depletion, light attenuation, and physical constraints of the growth substrate—would inevitably halt further biomass accumulation [50]. The attached-biomass nature of the twin-layer system adds an additional layer of complexity, as vertical biofilm thickening would eventually reduce light penetration and slow down photosynthetic efficiency. Moreover, from a physical point of view, there must be a maximum surface density threshold above which the gravitational force exerted on the algal biofilm exceeds the adhesive forces between the polycarbonate substrate and the biofilm, which behaves as a high-density fluid matrix [51]. This would cause the biofilm to 'drip' off the vertical panel, necessitating biomass collection before this occurs.

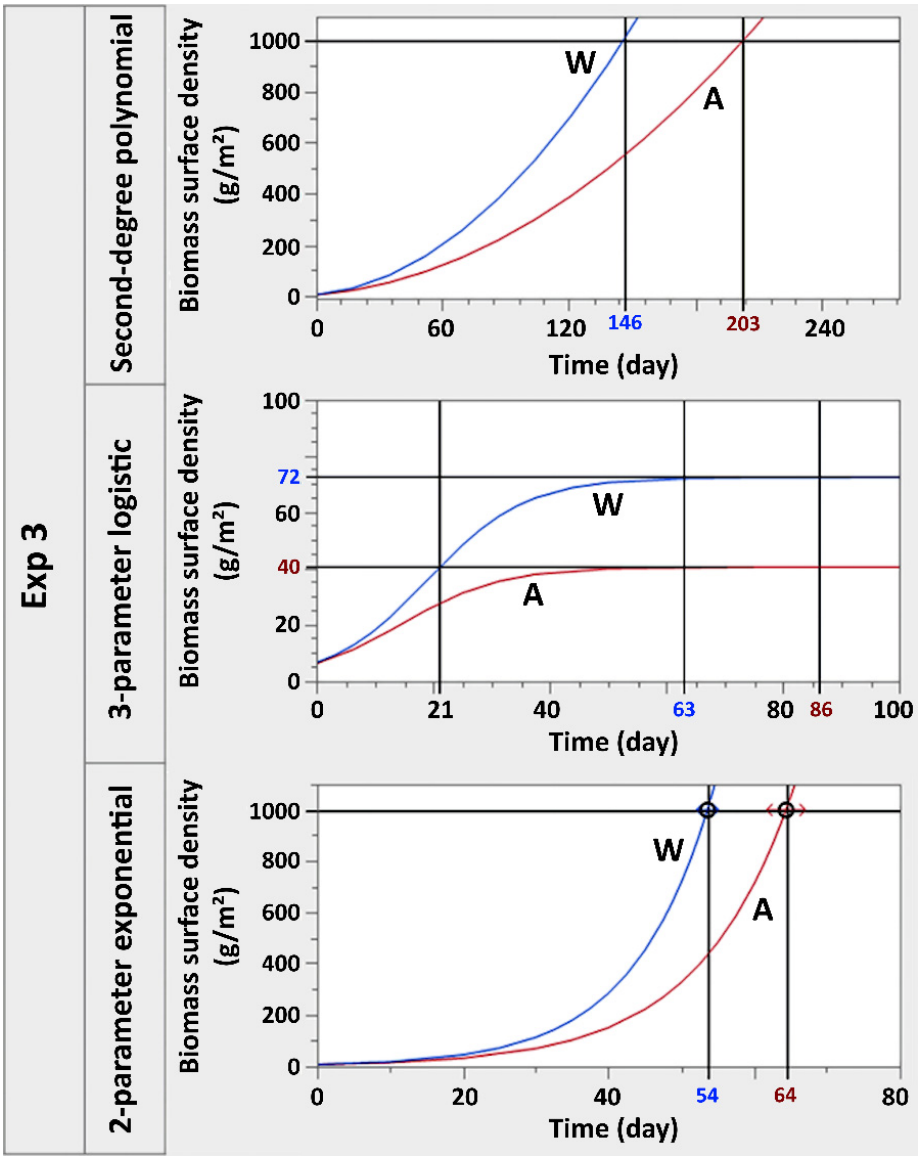

Figure 9. Regression curves for W and A during Exp3, with extended scales to highlight the time required to reach specific surface biomass density values.

As suggested, intrinsic phenomena related to biofilm stratification must be considered. Indeed, the dense biomass layer can be viewed as a stratification of cellular layers, with some layers more in contact with the internal interface, receiving more moisture and nutrients through the polycarbonate membrane, while other layers are more exposed to the atmospheric environment, promoting better gas exchange and more efficient light exposure [50]. As the biofilm thickens due to cell reproduction, the inner layers become progressively less efficient in terms of light, gas exchange and nutrient supply [51]. This progressive decrease in efficiency for the inner layers might correlate with a slowdown in growth rates after a certain period, thus necessitating the use of more complex and comprehensive mathematical models [50,52]. For instance, Li et al. [53] employed an automata model to investigate the behaviour of *Chlorella vulgaris* (Chlorophyta) algal biofilms under varying environmental and nutrient conditions. Their study highlighted two key findings: first, the existence of a critical depth (30–70 µm in their case) beyond which light penetration is insufficient, resulting in an active growth zone where cells actively engage in photosynthesis and contribute to biofilm thickening. Second, carbon

transport occurs with lower resistance through the deeper biofilm layers, where cellular respiration predominates.

Building on these considerations, future investigations with this cultivation system will benefit from monitoring actual growth rates over extended periods in order to, first of all, understand whether there really is a growth-inhibiting effect linked to the continuous supply of fresh medium as a stress condition, but more importantly to efficiently schedule biomass removal from the reactor as well as accurately quantify biomass production per unit area over a reference period (e.g., one year). Additionally, these tests will help assess the long-term durability of the material used as the source layer. Future research could also explore the use of environmentally friendly materials in place of the fibreglass used in this study, potentially utilising innovative polymers or structural materials derived from recycled waste [54].

3.3. Opportunities for Process Scale-Up and Use of Biomass Produced

The experimental results obtained under controlled laboratory conditions allow us to outline realistic scenarios for the scale-up of the twin-layer photobioreactor (TL-PBR) system, as well as to assess the potential applications and economic value of the algal biomass produced. From an operational standpoint, the TL-PBR demonstrated a low energy footprint, as the main power consumption was limited to the peristaltic pump, artificial illumination, and the sensor system. In a future pilot or full-scale configuration, artificial lighting could be replaced with natural sunlight, and the overall energy demand could be met using photovoltaic systems. A representative scale-up scenario might involve a cultivation surface of 100 m^2, achievable through the modular arrangement of ten 2 × 5 m panels. Under the most favourable experimental conditions observed (Exp3), regression analysis suggested that a surface biomass density of 1 kg_{DW}/m^2 could be reached in approximately 54 days, aligning with relevant literature that testifies to rates of 10–15 g m^{-2} day^{-1} [20]. This would correspond to a theoretical yield of 100 kg of dry, clean algal biomass in under two months.

However, this projection should be interpreted with caution, as it is derived from short-term lab trials and does not account for limiting factors such as non-uniform light distribution, accumulation of metabolic byproducts, or fouling. Large-scale cultivation would require addressing additional challenges such as the mechanical durability of the support materials, biofilm overgrowth, and long-term system maintenance. Biofilm thickness must be controlled to ensure adequate light penetration and gas exchange, and periodic harvesting is necessary to maintain productivity [53]. Repeated cleaning and scraping procedures may introduce physical stress to the materials used for the support and substrate layers, which could affect performance over time [55].

One particularly critical consideration when envisioning real-world applications is the method of sterilising the culture medium. In laboratory tests, sterilisation was achieved via autoclaving; however, this approach is impractical and unsustainable on a larger scale. In scaled-up systems, sterilisation could instead be implemented using UV lamps placed along the fluid circuit. This method is compatible with continuous or semicontinuous wastewater recirculation and has the advantage of being energy-efficient and reagent-free [56]. Nevertheless, it requires careful material selection, since the acidic pH used here in the cultivation of *G. phlegrea* (2.5) can cause corrosion in metallic components, especially in the collection tanks of standard UV units. For this reason, future configurations may evaluate the cultivation of *G. phlegrea* at slightly higher pH values, such as 4 to 5, which remain within the physiological tolerance of the species while mitigating corrosive effects and reducing the chemical demand associated with pH adjustment of the influent.

Another important factor to consider in scale-up scenarios is the variability of the wastewater composition. Unlike synthetic media, urban effluents exhibit seasonal and diurnal fluctuations in nutrient content, ionic strength, and the presence of potential inhibitors [24]. These variations can affect not only biomass growth rates but also biomass composition. The monitoring system implemented in our prototype—based on an IoT platform for real-time remote acquisition of environmental parameters such as temperature, pH, and light intensity—offers a useful foundation for adaptive system management [57–59]. Future iterations may benefit from the inclusion of additional sensors for nutrient concentrations or biofilm thickness, enabling closed-loop feedback control and predictive operation supported by artificial intelligence models [60–62].

From an economic and environmental perspective, the TL-PBR system shows advantages when compared with conventional systems such as open raceway ponds or tubular photobioreactors. Open ponds are characterised by low capital costs and simple operation, but they suffer from low areal productivity, high water losses through evaporation, and high risks of contamination. Tubular PBRs offer better control over culture conditions and higher volumetric productivity, but they require significant energy inputs for mixing, pumping, and often cooling, in addition to high infrastructure costs. Based on literature data, the operational cost of biomass production in open raceway ponds ranges approximately from EUR 2 to EUR 15 per kg of dry biomass [63–65], while closed PBRs can exceed EUR 30/kg under suboptimal conditions [66–68]. In contrast, TL-PBRs operate without active mixing, with minimal water use, and allow direct biomass harvesting via scraping, thereby avoiding the need for flocculation, filtration, or centrifugation—steps that are energetically expensive and economically burdensome [69,70]. Although no dedicated techno-economic study on TL-PBRs exists to date, these features suggest that operational costs could reasonably fall in the range of EUR 4 to EUR 10 per kg of dry biomass under realistic conditions, depending on scale, climate, and configuration. These data are inferential estimates based on significant overall cost reductions (over 50%) associated with lower harvesting costs, media production and mixing when using twin layers compared to other conventional cultivation systems [64,71–74].

The quality and purity of the biomass obtained represent another significant advantage [8,75]. As the TL-PBR supports attached growth in a controlled and physically isolated environment, the harvested biofilm is free of suspended solids and exhibits low microbial contamination. This clean biomass can serve as a platform for the production of high-value compounds, and the microalgal biomass of Galdieriaceae is a rich reservoir of biotechnologically relevant compounds, with an established market demand [1,2,33,76]. Literature reports indicate that members of the *Galdieria* genus can accumulate phycobiliproteins, particularly phycocyanin and allophycocyanin, with yields reaching up to 14% of dry weight [16]. Market prices for purified phycocyanin currently range from USD 500 to over 900 per kg, depending on purity and intended use [2,76]. In addition, *G. phlegrea* is known to produce carotenoids such as zeaxanthin and β-carotene, bioactive polysaccharides with antioxidant and immunomodulatory properties, sulphur- and nitrogen-rich amino acids relevant for feed applications, and polyunsaturated fatty acids including linolenic and arachidonic acid [1,11,33,77]. These compounds are in growing demand across the nutraceutical, cosmetic, and pharmaceutical sectors [8,74,76].

Even after the extraction of these compounds, the residual biomass can be further valorised. It may serve as feedstock for hydrothermal liquefaction processes [78] aimed at producing biofuels or be used as a biosorbent in processes for recovering metals and rare earth elements from industrial effluents [10,79,80]. These cascading uses align well with the principles of circular bioeconomy and are of increasing relevance in the context of EU strategies for waste valorisation and sustainable resource use [81,82].

Although the data presented here are based on short-term experiments conducted at laboratory scale, they provide an evidence-based foundation for future studies. The TL-PBR system, in its current configuration, combines low energy consumption, reduced water demand, simplified harvesting, and a capacity to produce high-purity biomass from low-cost inputs such as urban wastewater. Its modular nature and compatibility with smart monitoring infrastructures make it a promising candidate for deployment in decentralised contexts where resource efficiency and environmental sustainability are priorities. Further research at pilot and industrial scale will be essential to validate the long-term performance, economic feasibility, and environmental benefits of the system under real-world conditions.

4. Conclusions

This system enables the efficient cultivation of *G. phlegrea* using municipal wastewater as the sole nutrient source, producing a dense, clean algal paste ready for use. The integration of isotopic analysis with physical methodologies proved to be a crucial tool and provided valuable insights into metabolic pathways and algal growth dynamics. With low operating costs, the system is simple and easily scalable in greenhouses. Additionally, the novel implementation of a customised IoT-based system for real-time remote monitoring increases practical feasibility and provides an essential tool for supporting companies in managing harvesting and maintenance operations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app15095220/s1.

Author Contributions: Conceptualisation, M.R.d.C., C.C. and C.L.; methodology, M.R.d.C., S.A., S.B. and C.L.; software, A.S., A.M. and C.V.; validation, M.R.d.C. and C.L.; formal analysis, M.R.d.C., S.A., S.B. and C.L.; investigation, M.R.d.C., S.A., C.G., I.G. and C.L.; resources, A.S., C.C., A.M., C.V. and C.L.; data curation, M.R.d.C.; writing—original draft preparation, M.R.d.C. and C.L.; writing—review and editing, M.R.d.C., S.A., S.B. and C.L.; visualisation, M.R.d.C., C.G. and C.L.; supervision, M.R.d.C., C.C. and C.L.; project administration, M.R.d.C., C.C. and C.L.; funding acquisition, M.R.d.C., C.C. and C.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data are contained within the article. For more statistical reports, see Supplementary Materials. For any other data not specifically indicated in the article, data may be available on request.

Acknowledgments: The study presented in this article is related to the research objectives of the two projects GLASS ('Green recovery of silicon from solar panels through innovative biometallurgical process mediated by microorganisms'—concluded) and PHYRE ('PHYcoREcycling'—ongoing), respectively, funded by the University of Campania 'Luigi Vanvitelli' and the Italian Ministry for the Environment and Energy Security, and which provided the materials used in the study. The authors would like to express their gratitude to Luciano di Cicco of Archimetal 2.0 Società Cooperativa and Davide Galetti and Fabio Corte of MINT s.r.l. for their invaluable technical assistance in the construction of the prototype's structural components and the development of the sensor apparatus. Further acknowledgement ought to be given to Luciano di Cicco for offering valuable linguistic revision assistance and to Concetta Andreozzi for improving the quality of the figures in the article. Finally, the corresponding author acknowledges the support of the European Union "PON Ricerca e Innovazione 2014–2020" initiative, in accordance with Article 24, paragraph 3, letter a) of Law no. 240 of 30 December 2010, and Ministerial Decree no. 1062 of 10 August 2021.

Conflicts of Interest: Author Antonio Spagnuolo was employed by Energreenup S.r.l. Author Carmela Vetromile was employed by Reverseup S.r.l. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

A	Allen medium
AICc	Akaike Information Criterion corrected
BIC	Bayesian Information Criterion
EA-IRMS	Elemental Analysis coupled with Isotopic Ratio Mass Spectrometry
IoT	Internet of Things
MSE	Mean Squared Error
PBR	Photobioreactor
RMSE	Root Mean Squared Error
SEM-EDS	Scanning Electron Microscopy coupled with Energy-Dispersive Spectroscopy
SSE	Sum of Squared Errors
TL	twin layers
TL-PBR	twin-layer type photobioreactor
W	wastewater medium

References

1. Canelli, G.; Abiusi, F.; Vidal Garcia, A.; Canziani, S.; Mathys, A. Amino acid profile and protein bioaccessibility of two *Galdieria sulphuraria* strains cultivated autotrophically and mixotrophically in pilot-scale photobioreactors. *Innov. Food Sci. Emerg. Technol.* **2023**, *84*, 103287. [CrossRef]
2. Jacob-Lopes, E.; Maroneze, M.M.; Deprá, M.C.; Sartori, R.B.; Dias, R.R.; Zepka, L.Q. Bioactive food compounds from microalgae: An innovative framework on industrial biorefineries. *Curr. Opin. Food Sci.* **2019**, *25*, 1–7. [CrossRef]
3. Show, K.-Y.; Yan, Y.; Zong, C.; Guo, N.; Chang, J.-S.; Lee, D.-J. State of the art and challenges of biohydrogen from microalgae. *Bioresour. Technol.* **2019**, *289*, 121747. [CrossRef]
4. González, I.; Herrero, N.; Siles, J.Á.; Chica, A.F.; Martín, M.; Izquierdo, C.G.; Gómez, J.M. Wastewater nutrient recovery using twin-layer microalgae technology for biofertilizer production. *Water Sci. Technol.* **2020**, *82*, 1044–1061. [CrossRef] [PubMed]
5. Iovinella, M.; Palmieri, M.; Papa, S.; Auciello, C.; Ventura, R.; Lombardo, F.; Race, M.; Lubritto, C.; di Cicco, M.R.; Davis, S.J.; et al. Biosorption of rare earth elements from luminophores by *G. sulphuraria* (Cyanidiophytina, Rhodophyta). *Environ. Res.* **2023**, *239*, 117281. [CrossRef] [PubMed]
6. Čížková, M.; Mezricky, P.; Mezricky, D.; Rucki, M.; Zachleder, V.; Vítová, M. Bioaccumulation of Rare Earth Elements from Waste Luminophores in the Red Algae, *Galdieria phlegrea*. *Waste Biomass Valorization* **2020**, *12*, 3137–3146. [CrossRef]
7. Yang, S.; Fan, Y.; Cao, Y.; Wang, Y.; Mou, H.; Sun, H. Technological readiness of commercial microalgae species for foods. *Crit. Rev. Food Sci. Nutr.* **2024**, *64*, 7993–8017. [CrossRef]
8. Gallego, I.; Medic, N.; Pedersen, J.S.; Ramasamy, P.K.; Robbens, J.; Vereecke, E.; Romeis, J. The microalgal sector in Europe: Towards a sustainable bioeconomy. *New Biotechnol.* **2025**, *86*, 1–13. [CrossRef]
9. Malavasi, V.; Soru, S.; Cao, G. Extremophile Microalgae: The potential for biotechnological application. *J. Phycol.* **2020**, *56*, 559–573. [CrossRef]
10. Palmieri, M.; Iovinella, M.; Davis, S.J.; di Cicco, M.R.; Lubritto, C.; Race, M.; Papa, S.; Fabbricino, M.; Ciniglia, C. *Galdieria sulphuraria* ACUF427 Freeze-Dried Biomass as Novel Biosorbent for Rare Earth Elements. *Microorganisms* **2022**, *10*, 2138. [CrossRef]
11. Retta, B.; Iovinella, M.; Ciniglia, C. Significance and Applications of the Thermo-Acidophilic Microalga *Galdieria sulphuraria* (Cyanidiophytina, Rhodophyta). *Plants* **2024**, *13*, 1786. [CrossRef] [PubMed]
12. Selvaratnam, T.; Pegallapati, A.K.; Montelya, F.; Rodriguez, G.; Nirmalakhandan, N.; Van Voorhies, W.; Lammers, P.J. Evaluation of a thermo-tolerant acidophilic alga, *Galdieria sulphuraria*, for nutrient removal from urban wastewaters. *Bioresour. Technol.* **2014**, *156*, 395–399. [CrossRef] [PubMed]
13. di Cicco, M.R.; Iovinella, M.; Palmieri, M.; Lubritto, C.; Ciniglia, C. Extremophilic Microalgae Galdieria Gen. for Urban Wastewater Treatment: Current State, the Case of "POWER" System, and Future Prospects. *Plants* **2021**, *10*, 2343. [CrossRef] [PubMed]
14. Henkanatte-Gedera, S.M.; Selvaratnam, T.; Karbakhshravari, M.; Myint, M.; Nirmalakhandan, N.; Van Voorhies, W.; Lammers, P.J. Removal of dissolved organic carbon and nutrients from urban wastewaters by *Galdieria sulphuraria*: Laboratory to field scale demonstration. *Algal Res.* **2017**, *24*, 450–456. [CrossRef]
15. di Cicco, M.R.; Palmieri, M.; Altieri, S.; Ciniglia, C.; Lubritto, C. Cultivation of the Acidophilic Microalgae *Galdieria phlegrea* with Wastewater: Process Yields. *Int. J. Environ. Res. Public Health* **2021**, *18*, 2291. [CrossRef]
16. Wan, M.; Wang, Z.; Zhang, Z.; Wang, J.; Li, S.; Yu, A.; Li, Y. A novel paradigm for the high-efficient production of phycocyanin from *Galdieria sulphuraria*. *Bioresour. Technol.* **2016**, *218*, 272–278. [CrossRef]

17. Fasaei, F.; Bitter, J.; Slegers, P.; Van Boxtel, A. Techno-economic evaluation of microalgae harvesting and dewatering systems. *Algal Res.* **2018**, *31*, 347–362. [CrossRef]
18. Tan, J.S.; Lee, S.Y.; Chew, K.W.; Lam, M.K.; Lim, J.W.; Ho, S.-H.; Show, P.L. A review on microalgae cultivation and harvesting, and their biomass extraction processing using ionic liquids. *Bioengineered* **2020**, *11*, 116–129. [CrossRef]
19. Acién, F.G.; Molina, E.; Fernández-Sevilla, J.M.; Barbosa, M.; Gouveia, L.; Sepúlveda, C.; Bazaes, J.; Arbib, Z. 20—Economics of microalgae production. In *Microalgae-Based Biofuels and Bioproducts*; Gonzalez-Fernandez, C., Muñoz, R., Eds.; Woodhead Publishing: Cambridge, UK, 2017; pp. 485–503.
20. Carbone, D.A.; Olivieri, G.; Pollio, A.; Melkonian, M. Biomass and phycobiliprotein production of *Galdieria sulphuraria*, immobilized on a twin-layer porous substrate photobioreactor. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 3109–3119. [CrossRef]
21. Naumann, T.; Çebi, Z.; Podola, B.; Melkonian, M. Growing microalgae as aquaculture feeds on twin-layers: A novel solid-state photobioreactor. *J. Appl. Phycol.* **2013**, *25*, 1413–1420. [CrossRef]
22. Muñoz, M.; Guzmán, J.L.; Torres, M.; Acién, F.G. An IoT platform for data management in an industrial-scale microalgae cultivation plant. *IEEE Access* **2022**, *10*, 127128–127139. [CrossRef]
23. Wang, K.; Khoo, K.S.; Leong, H.Y.; Nagarajan, D.; Chew, K.W.; Ting, H.Y.; Selvarajoo, A.; Chang, J.-S.; Show, P.L. How does the Internet of Things (IoT) help in microalgae biorefinery? *Biotechnol. Adv.* **2022**, *54*, 107819. [CrossRef] [PubMed]
24. di Cicco, M.R.; Masiello, A.; Spagnuolo, A.; Vetromile, C.; Borea, L.; Giannella, G.; Iovinella, M.; Lubritto, C. Real-Time Monitoring and Static Data Analysis to Assess Energetic and Environmental Performances in the Wastewater Sector: A Case Study. *Energies* **2021**, *14*, 6948. [CrossRef]
25. Aron, N.S.M.; Khoo, K.S.; Chew, K.W.; Veeramuthu, A.; Chang, J.-S.; Show, P.L. Microalgae cultivation in wastewater and potential processing strategies using solvent and membrane separation technologies. *J. Water Process Eng.* **2021**, *39*, 101701.
26. Pinto, G.; Ciniglia, C.; Cascone, C.; Pollio, A. Species Composition of Cyanidiales Assemblages in Pisciarelli (Campi Flegrei, Italy) and Description of *Galdieria phlegrea* SP. NOV. In *Algae and Cyanobacteria in Extreme Environments*; Seckbach, J., Ed.; Springer: Dordrecht, The Netherlands, 2007; pp. 487–502.
27. Zuccaro, G.; Yousuf, A.; Pollio, A.; Steyer, J.-P. Chapter 2—Microalgae Cultivation Systems. In *Microalgae Cultivation for Biofuels Production*; Yousuf, A., Ed.; Academic Press: Cambridge, MA, USA, 2020; pp. 11–29.
28. Altieri, S.; Saiano, K.; Biondi, M.; Ricci, P.; Lubritto, C. Traceability of 'Mozzarella di Bufala Campana'production chain by means of carbon, nitrogen and oxygen stable isotope ratios. *J. Sci. Food Agric.* **2020**, *100*, 995–1003. [CrossRef]
29. Criss, R.E. *Principles of Stable Isotope Distribution*; Oxford University Press: Oxford, UK, 1999.
30. Germinario, C.; Cultrone, G.; Cavassa, L.; De Bonis, A.; Izzo, F.; Langella, A.; Mercurio, M.; Morra, V.; Munzi, P.; Grifa, C. Local production and imitations of Late Roman pottery from a well in the Roman necropolis of Cuma in Naples, Italy. *Geoarchaeology* **2019**, *34*, 62–79. [CrossRef]
31. Yousuf, A. Fundamentals of microalgae cultivation. In *Microalgae Cultivation for Biofuels Production*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 1–9.
32. Varshney, P.; Sohoni, S.; Wangikar, P.P.; Beardall, J. Effect of high CO_2 concentrations on the growth and macromolecular composition of a heat- and high-light-tolerant microalga. *J. Appl. Phycol.* **2016**, *28*, 2631–2640. [CrossRef]
33. Abiusi, F.; Moñino Fernández, P.; Canziani, S.; Janssen, M.; Wijffels, R.H.; Barbosa, M. Mixotrophic cultivation of *Galdieria sulphuraria* for C-phycocyanin and protein production. *Algal Res.* **2022**, *61*, 102603. [CrossRef]
34. Wang, H.; Zhang, Z.; Wan, M.; Wang, R.; Huang, J.; Zhang, K.; Guo, J.; Bai, W.; Li, Y. Comparative study on light attenuation models of *Galdieria sulphuraria* for efficient production of phycocyanin. *J. Appl. Phycol.* **2020**, *32*, 165–174. [CrossRef]
35. Carbone, D.A.; Olivieri, G.; Pollio, A.; Melkonian, M. Comparison of Galdieria growth and photosynthetic activity in different culture systems. *AMB Express* **2020**, *10*, 170. [CrossRef]
36. Sloth, J.K.; Wiebe, M.G.; Eriksen, N.T. Accumulation of phycocyanin in heterotrophic and mixotrophic cultures of the acidophilic red alga *Galdieria sulphuraria*. *Enzym. Microb. Technol.* **2006**, *38*, 168–175. [CrossRef]
37. Buckeridge, E.; Caballero, C.C.; Smith, D.H.; Stott, M.B.; Carere, C.R. Substrate and nutrient manipulation during continuous cultivation of extremophilic algae, *Galdieria* spp. RTK 37.1, substantially impacts biomass productivity and composition. *Biotechnol. Bioeng.* **2024**, *121*, 3428–3439. [CrossRef]
38. NOAA. NOAA Earth System Research Laboratories. Available online: https://www.esrl.noaa.gov/ (accessed on 11 June 2024).
39. di Cicco, M.R.; Altieri, S.; Mantile, N.; Petitti, P.; Persiani, C.; Conti, A.M.; Allegrezza, L.; Cavazzuti, C.; Lubritto, C. Exploring Burial and Dietary Patterns at the Copper Age Necropolis of Selvicciola (Viterbo, Italy): New Perspectives from 14C and Stable Isotope Data. *Heritage* **2024**, *7*, 3291–3309. [CrossRef]
40. Mohd Udaiyappan, A.F.; Hasan, H.A.; Takriff, M.S.; Abdullah, S.R.S.; Maeda, T.; Mustapha, N.A.; Mohd Yasin, N.H.; Nazashida Mohd Hakimi, N.I. Microalgae-bacteria interaction in palm oil mill effluent treatment. *J. Water Process Eng.* **2020**, *35*, 101203. [CrossRef]
41. Iovinella, M.; Carbone, D.A.; Diana, C.; Seth, J.D.; Michele, I.; Esposito, S.; Ciniglia, C. Prevalent pH Controls the Capacity of Galdieria maxima to Use Ammonia and Nitrate as a Nitrogen Source. *Plants* **2020**, *9*, 232. [CrossRef] [PubMed]

42. Bateman, A.S.; Kelly, S.D. Fertilizer nitrogen isotope signatures. *Isot. Environ. Health Stud.* **2007**, *43*, 237–247. [CrossRef]
43. Su, Y. Revisiting carbon, nitrogen, and phosphorus metabolisms in microalgae for wastewater treatment. *Sci. Total Environ.* **2021**, *762*, 144590.
44. Azuara, M.P.; Aparicio, P.J. In Vivo Blue-Light Activation of Chlamydomonas reinhardii Nitrate Reductase 1. *Plant Physiol.* **1983**, *71*, 286–290. [CrossRef]
45. Salbitani, G.; Carfagna, S. Ammonium Utilization in Microalgae: A Sustainable Method for Wastewater Treatment. *Sustainability* **2021**, *13*, 956. [CrossRef]
46. Sloth, J.K.; Jensen, H.C.; Pleissner, D.; Eriksen, N.T. Growth and phycocyanin synthesis in the heterotrophic microalga *Galdieria sulphuraria* on substrates made of food waste from restaurants and bakeries. *Bioresour. Technol.* **2017**, *238*, 296–305. [CrossRef]
47. Bellido-Pedraza, C.M.; Calatrava, V.; Sanz-Luque, E.; Tejada-Jiménez, M.; Llamas, Á.; Plouviez, M.; Guieysse, B.; Fernández, E.; Galván, A. Chlamydomonas reinhardtii, an Algal Model in the Nitrogen Cycle. *Plants* **2020**, *9*, 903. [CrossRef] [PubMed]
48. Sun, Z.; Xi, J.; Yang, R.; Lu, L.; Qiu, W.; Yang, B. Structures and compositions of biofilms in moving bed biofilm reactors pretreated by four drying methods. *Chem. Eng. J.* **2023**, *477*, 147228. [CrossRef]
49. Sun, Y.; Shi, M.; Lu, T.; Ding, D.; Sun, Y.; Yuan, Y. Bio-removal of PtCl62− complex by *Galdieria sulphuraria*. *Sci. Total Environ.* **2021**, *796*, 149021. [CrossRef] [PubMed]
50. Wang, J.-H.; Zhuang, L.-L.; Xu, X.-Q.; Deantes-Espinosa, V.M.; Wang, X.-X.; Hu, H.-Y. Microalgal attachment and attached systems for biomass production and wastewater treatment. *Renew. Sustain. Energy Rev.* **2018**, *92*, 331–342.
51. Polizzi, B.; Bernard, O.; Ribot, M. A time-space model for the growth of microalgae biofilms for biofuel production. *J. Theor. Biol.* **2017**, *432*, 55–79. [CrossRef] [PubMed]
52. Huang, J.; Chu, R.; Chang, T.; Cheng, P.; Jiang, J.; Yao, T.; Zhou, C.; Liu, T.; Ruan, R. Modeling and improving arrayed microalgal biofilm attached culture system. *Bioresour. Technol.* **2021**, *331*, 124931.
53. Li, L.; Wang, Y.; Gao, L.; Zhou, W.; Chen, L.; Zhang, Z.; Liu, T. Experiments and cellular automata simulation reveal light/carbon transportation and growth mechanism of Chlorella vulgaris biofilm in attached cultivation. *Chem. Eng. J.* **2023**, *457*, 141177. [CrossRef]
54. de Assis, L.R.; Calijuri, M.L.; Assemany, P.P.; Berg, E.C.; Febroni, L.V.; Bartolomeu, T.A. Evaluation of the performance of different materials to support the attached growth of algal biomass. *Algal Res.* **2019**, *39*, 101440. [CrossRef]
55. Murshid, S.; Antonysamy, A.; Dhakshinamoorthy, G.; Jayaseelan, A.; Pugazhendhi, A. A review on biofilm-based reactors for wastewater treatment: Recent advancements in biofilm carriers, kinetics, reactors, economics, and future perspectives. *Sci. Total Environ.* **2023**, *892*, 164796. [CrossRef]
56. Yu, L.; Acosta, N.; Bautista, M.A.; McCalder, J.; Himann, J.; Pogosian, S.; Hubert, C.R.; Parkins, M.D.; Achari, G. Quantitative evaluation of municipal wastewater disinfection by 280 nm UVC LED. *Water* **2023**, *15*, 1257. [CrossRef]
57. Havlik, I.; Lindner, P.; Scheper, T.; Reardon, K.F. On-line monitoring of large cultivations of microalgae and cyanobacteria. *Trends Biotechnol.* **2013**, *31*, 406–414. [CrossRef]
58. Sandnes, J.M.; Ringstad, T.; Wenner, D.; Heyerdahl, P.H.; Källqvist, T.; Gislerød, H.R. Real-time monitoring and automatic density control of large-scale microalgal cultures using near infrared (NIR) optical density sensors. *J. Biotechnol.* **2006**, *122*, 209–215. [CrossRef] [PubMed]
59. Longo, M.; Rioual, S.; Talbot, P.; Faÿ, F.; Hellio, C.; Lescop, B. A high sensitive microwave sensor to monitor bacterial and biofilm growth. *Sens. Bio-Sens. Res.* **2022**, *36*, 100493. [CrossRef]
60. Porras Reyes, L.; Havlik, I.; Beutel, S. Software sensors in the monitoring of microalgae cultivations. *Rev. Environ. Sci. Bio/Technol.* **2024**, *23*, 67–92. [CrossRef]
61. Xu, K.; Zhu, Z.; Yu, H.; Zou, X. Prediction of microalgae harvesting efficiency and identification of important parameters for ballasted flotation using an optimized machine learning model. *Algal Res.* **2025**, *87*, 103985. [CrossRef]
62. Syed, T.; Krujatz, F.; Ihadjadene, Y.; Mühlstädt, G.; Hamedi, H.; Mädler, J.; Urbas, L. A review on machine learning approaches for microalgae cultivation systems. *Comput. Biol. Med.* **2024**, *172*, 108248. [CrossRef]
63. Norsker, N.-H.; Barbosa, M.J.; Vermuë, M.H.; Wijffels, R.H. Microalgal production—A close look at the economics. *Biotechnol. Adv.* **2011**, *29*, 24–27. [CrossRef]
64. Slade, R.; Bauen, A. Micro-algae cultivation for biofuels: Cost, energy balance, environmental impacts and future prospects. *Biomass Bioenergy* **2013**, *53*, 29–38. [CrossRef]
65. Chen, M.; Chen, Y.; Zhang, Q. A Review of Energy Consumption in the Acquisition of Bio-Feedstock for Microalgae Biofuel Production. *Sustainability* **2021**, *13*, 8873. [CrossRef]
66. Quinn, J.C.; Davis, R. The potentials and challenges of algae based biofuels: A review of the techno-economic, life cycle, and resource assessment modeling. *Bioresour. Technol.* **2015**, *184*, 444–452. [CrossRef]
67. Davis, R.; Markham, J.; Kinchin, C.; Grundl, N.; Tan, E.C.; Humbird, D. *Process Design and Economics for the Production of Algal Biomass: Algal Biomass Production in Open Pond Systems and Processing Through Dewatering for Downstream Conversion*; National Renewable Energy Laboratory(NREL): Golden, CO, USA, 2016.

68. Manninen, K.; Sonck, M.; Spilling, K. *Environmental Impact Assessment of Algae Cultivation Under Finnish Conditions*; CLEEN Ltd.: Helsinki, Finland, 2013.
69. Yin, Z.; Zhu, L.; Li, S.; Hu, T.; Chu, R.; Mo, F.; Hu, D.; Liu, C.; Li, B. A comprehensive review on cultivation and harvesting of microalgae for biodiesel production: Environmental pollution control and future directions. *Bioresour. Technol.* **2020**, *301*, 122804. [CrossRef]
70. Bhatia, S.K.; Mehariya, S.; Bhatia, R.K.; Kumar, M.; Pugazhendhi, A.; Awasthi, M.K.; Atabani, A.E.; Kumar, G.; Kim, W.; Seo, S.-O.; et al. Wastewater based microalgal biorefinery for bioenergy production: Progress and challenges. *Sci. Total Environ.* **2021**, *751*, 141599. [CrossRef]
71. Vázquez-Romero, B.; Perales, J.A.; Pereira, H.; Barbosa, M.; Ruiz, J. Techno-economic assessment of microalgae production, harvesting and drying for food, feed, cosmetics, and agriculture. *Sci. Total Environ.* **2022**, *837*, 155742. [CrossRef]
72. Bhatt, A.; Khanchandani, M.; Rana, M.S.; Prajapati, S.K. Techno-economic analysis of microalgae cultivation for commercial sustainability: A state-of-the-art review. *J. Clean. Prod.* **2022**, *370*, 133456. [CrossRef]
73. Ennaceri, H.; Ishika, T.; Mkpuma, V.O.; Moheimani, N.R. Microalgal biofilms: Towards a sustainable biomass production. *Algal Res.* **2023**, *72*, 103124. [CrossRef]
74. Loke Show, P. Global market and economic analysis of microalgae technology: Status and perspectives. *Bioresour. Technol.* **2022**, *357*, 127329. [CrossRef]
75. Barsanti, L.; Gualtieri, P. Is exploitation of microalgae economically and energetically sustainable? *Algal Res.* **2018**, *31*, 107–115. [CrossRef]
76. Benavente-Valdés, J.R.; Rodríguez-Zúñiga, D.; Cepeda-Tovar, V.; Solís-Quiroz, O. Commercial Compounds from Algae. In *Microbial Bioactive Compounds: Industrial and Agricultural Applications*; Soni, R., Suyal, D.C., Morales-Oyervides, L., Eds.; Springer Nature: Cham, Switzerland, 2023; pp. 37–58.
77. Hirooka, S.; Miyagishima, S.-Y. Cultivation of acidophilic algae *Galdieria sulphuraria* and *Pseudochlorella* sp. YKT1 in media derived from acidic hot springs. *Front. Microbiol.* **2016**, *7*, 2022. [CrossRef]
78. Fan, Q.; Fu, P.; Song, C.; Fan, Y. Valorization of waste biomass through hydrothermal liquefaction: A review with focus on linking hydrothermal factors to products characteristics. *Ind. Crops Prod.* **2023**, *191*, 116017. [CrossRef]
79. Iovinella, M.; Lombardo, F.; Ciniglia, C.; Palmieri, M.; di Cicco, M.R.; Trifuoggi, M.; Race, M.; Manfredi, C.; Lubritto, C.; Fabbricino, M.; et al. Bioremoval of Yttrium (III), Cerium (III), Europium (III), and Terbium (III) from Single and Quaternary Aqueous Solutions Using the Extremophile *Galdieria sulphuraria* (Galdieriaceae, Rhodophyta). *Plants* **2022**, *11*, 1376. [CrossRef] [PubMed]
80. Brown, R.M.; Mirkouei, A.; Reed, D.; Thompson, V. Current nature-based biological practices for rare earth elements extraction and recovery: Bioleaching and biosorption. *Renew. Sustain. Energy Rev.* **2023**, *173*, 113099. [CrossRef]
81. Anuardo, R.G.; Espuny, M.; Costa, A.C.F.; Espuny, A.L.G.; Kazançoğlu, Y.; Kandsamy, J.; de Oliveira, O.J. Transforming E-Waste into Opportunities: Driving Organizational Actions to Achieve Sustainable Development Goals. *Sustainability* **2023**, *15*, 14150. [CrossRef]
82. Shittu, O.S.; Williams, I.D.; Shaw, P.J. Global E-waste management: Can WEEE make a difference? A review of e-waste trends, legislation, contemporary issues and future challenges. *Waste Manag.* **2021**, *120*, 549–563. [CrossRef]