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Integrated Microalgal Wastewater Treatment and Biomass Valorization: A Circular Economy Approach Using Chlorella sorokiniana and Scenedesmus obliquus.

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Integrated Microalgal Wastewater Treatment and Biomass Valorization: A Circular Economy Approach Using *Chlorella sorokiniana* and *Scenedesmus obliquus*.

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Abstract

This study evaluates the potential of two green microalgal strains, *Chlorella sorokiniana* and *Scenedesmus obliquus*, for the treatment of primary urban wastewater coupled with the production of value-added biomass. Cultivated in semi-continuous mode at a dilution rate of 0.3 day^{-1}, both species demonstrated high efficiency in removing nutrients and contaminants. Significant reductions were achieved for nitrates, ammonium, phosphates, and heavy metals (Fe^{2+}, Cr, $Ni^{2+}$and Cu^{2+}) with removal efficiencies ranging from 19% to 100%. These results confirm that the treated effluent complies with regulatory standards for agricultural water reuse. Biochemical characterization revealed distinct metabolic profiles between the strains: *C. sorokiniana* was predominantly protein-rich (43.40% of dry weight), whereas *S. obliquus* exhibited a higher carbohydrate content (39.07%). Within a circular economy framework, aqueous extracts obtained via sonication were assessed for their biostimulant potential. *C. sorokiniana* extracts showed superior performance, increasing the germination index of watercress seeds by up to 135% at 0.5 $g \cdot L^{-1}$. Additionally, this strain enhanced adventitious root formation in soybean by 129% and 218% at concentrations of 0.5 and 2.0 $g \cdot L^{-1}$, respectively. It also exhibited cytokinin-like activity in cucumber cotyledons and uniquely promoted chlorophyll retention in wheat leaves. The results demonstrate that microalgae-based treatment of primary urban wastewater represents an effective and sustainable dual-purpose strategy. This approach enables efficient bioremediation for water reclamation while generating high-value biomass suitable for agricultural applications, thereby supporting the transition toward a bio-based circular economy.

Keywords: Biomass; Biostimulants; Microalgae; Nutrient removal; Primary wastewater; Sustainability; Wastewater reuse.

1. Introduction

Increasingly stringent environmental regulations reflect the critical global challenge of mitigating pollution to safeguard public health and economic stability. Despite high-level discourse on sustainable water management, freshwater resources remain under systemic threat. Rapid industrialization and accelerating economic growth continue to compromise both the availability and quality of water, necessitating urgent advancements in water reclamation strategies [1]. Approximately 30% of the global freshwater supply is consumed by industries and urban areas, which generate large volumes of wastewater, containing a wide range of chemicals at varying concentrations [2] [3] urban wastewaters (UWW) include human waste, urine, organic substances, and sludge[4].

Urban wastewater typically contains biodegradable organic material, nutrients, hormones, heavy metals, pharmaceuticals, detergents, oils, pesticides, fats, pathogenic microorganisms, and other contaminants. High concentrations of inorganic nutrients, such as nitrogen (N) in the form of ammonium and phosphorus (P) as orthophosphates, result from the decomposition of urea and proteins, as well as microbial breakdown of organic phosphates and polyphosphates. [3] Untreated. wastewater can cause the eutrophication of aquatic ecosystems, posing significant risks to water quality. Therefore, it is essential to implement effective treatment strategies to reduce and eliminate pollutants such as ammonium (NH_4^+), nitrate (NO_3^-), and phosphate (PO_4^{3-}). These measures are crucial for the preservation of aquatic environments. [5] However, traditional methods for nutrient removal are constrained by their high energy demands and environmental repercussions, such as greenhouse gas emissions. Additionally, these methods face difficulties in complying with increasingly stringent nutrient discharge regulations. Given these limitations, the demand for innovative and environmentally sustainable alternatives to replace traditional treatment methods is increasing [6]**.**

Microalgae-based systems represent a highly promising and sustainable approach for advanced wastewater purification [7]. These photosynthetic microorganisms excel in nutrient-enriched media, demonstrating an exceptional capacity for CO_2 fixation and the removal of organic matter, xenobiotics, and heavy metals [3], [8]. Integrating microalgae as a tertiary treatment step in wastewater treatment plants (WWTPs) offers a cost-effective method for nutrient (*N* and *P*) removal, facilitating water reuse for fertigation according to EU irrigation standards while minimizing environmental impacts [3][5]**.** The design of the bioreactor is critical, although closed photobioreactors (PBRs) achieve higher biomass productivity than open systems, their elevated capital and energy requirements significantly impact overall costs, then the use of open

raceway reactors is the most extended solution [9] . In these systems, a consortium of microalgae and bacteria develops; however, when properly managed, microalgae typically become the dominant microorganisms. Among them, Chlorella and Scenedesmus are the most frequently observed genera. The success of the process depends on evaluating each strain's tolerance to specific wastewater conditions, as well as its light-use efficiency and nutrient requirements [10].

Beyond phytoremediation, this process generates valuable biomass for non-human related applications such as bioenergy, materials and agricultural applications [3]. In agriculture, microalgae can be processed to obtain both biopesticides and biostimulants, with the latter being the most widely used. The biostimulant properties of microalgal extracts are mainly attributed to their primary metabolites, including proteins, carbohydrates, lipids, and essential amino acids such as arginine and proline [11]. Furthermore, the presence of polysaccharides and phytohormones (auxins, gibberellins, and cytokinins), even in trace amounts, exerts significant physiological effects that lead to notable agronomic benefits [12].

This research aims to evaluate the secondary treatment of real urban wastewater employing selected microalgal strains. The research follows a multi-faceted approach: (i) assessing water reclamation prospects relative to legal standards, (ii) characterizing the biostimulant potential of the resulting biomass, and (iii) evaluating the overall environmental impact of the process. By integrating wastewater remediation with biomass valorization, this work operates within a circular economy framework. It aligns with the European Commission's Farm to Fork Strategy (2020) to promote sustainable food systems, while maintaining the critical regulatory distinction between biostimulants and biofertilizers.

2. Materials and methods

2.1. Wastewater composition

Raw domestic wastewater, including blackwater and greywater components [13], was sourced from the University of Almería (Spain). The effluent was utilized without conventional depuration, following only a preliminary solids removal step. Samples were collected at the start of the experimental period and cryopreserved at -20°C in clean plastic bottles to prevent any compositional alterations prior to microalgal cultivation and bioremediation experiments.

2.2. Microorganisms and Inoculum Preparation

The microalgal strains *Chlorella sorokiniana* and *Scenedesmus obliquus* were obtained from the culture collection of the Department of Chemical Engineering at the University of Almería (Fig. 1). These species were selected for their rapid growth rates, established performance in wastewater treatment [13], and previously documented phytostimulant potential [14], [15]. Inocula were prepared in batch mode using a modified Arnon medium [16] in 1,000 mL Erlenmeyer flasks. Cultures were maintained at room temperature (23°C ± 2°C) under a 12:12 h light/dark photoperiod. Artificial lighting was provided by 90 W LED fluorescent lamps at a photosynthetic photon flux density (PPFD) of 200 µE m^{-2} s^{-1} (verified by a ROLINE RO 1332 A digital luxmeter). Constant agitation and aeration were ensured by an air flow of 15.2 L. h^{-1} (Stellar 380D pump), with flasks sealed with hydrophobic cotton to maintain sterility.

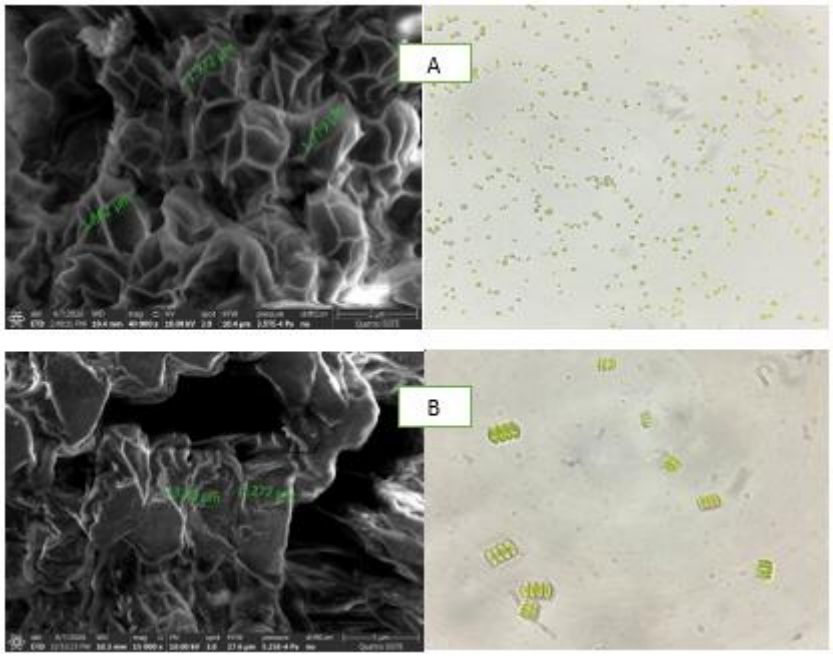


Fig.1: Strains of *C. sorokiniana* (A) and *S. obliquus* (B). The images were acquired using scanning electron microscopy (SEM, left) and binocular microscopy (right).

2.3. Bioreactor Configuration and Experimental Conditions

Cultivation was performed in bubble-column photobioreactors (PBRs) featuring spherical bases, with a height of 45 cm, a diameter of 3 cm, and a working volume of 0.25 L (Fig. 2). For both *S. obliquus* and *C. sorokiniana*, experiments were conducted in triplicate for two distinct

conditions: a control group and a treatment group supplied with untreated primary urban wastewater (PTWW).

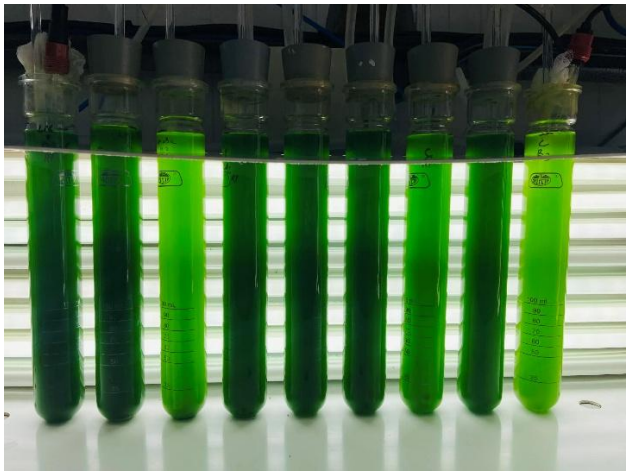


Fig 2: Bubble-column photobioreactors (PBRs)

The control medium was formulated with 0.18 g. L^{-1}, $MgSO_4$ 0.14 g L^{-1}, K_2PO_4 0.90 g. L^{-1}, $NaNO_3$, and 0.03 g L^{-1} of Karentol® (Kenogard, Spain), a commercial micronutrient mixture. The raw wastewater was sourced from an urban treatment plant in Almería, Spain, characterized by initial concentrations of 145.3 mg. L^{-1}, NH_4^+ 1.53 mg L^{-1}, NO_3^- 9.73 mg L^{-1}, PO_4^{3-} and 220 mg L^{-1}Chemical Oxygen Demand (COD).

To ensure mixing and prevent dissolved oxygen accumulation, The reactors were aerated at a flow rate of 0.2 v/v/min. Temperature was stabilized at 20°C within a climate-controlled chamber. The pH was strictly remained at 8.0 ± 0.1 through an automated on-demand CO_2 injection system into the air stream, regulated by solenoid valves and pH sensors. Illumination was provided by eight high-efficiency fluorescent tubes (Philips Daylight T5, 28 W) placed 4 cm from the PBRs. A circadian cycle was simulated, featuring a progressive light ramp from 08:00 to 14:00 h, followed by a decrease until 20:00 h. The maximum Photosynthetically Active Radiation (PAR) inside the columns (in the absence of cells) was measured at 1,857 $\mu Em^{-2}s^{-1}$, using an SQS-100 spherical quantum sensor (Walz GmbH, Germany).

All reactors were simultaneously inoculated at 10% (v/v). Following a 7-day batch phase, the system transitioned to semi-continuous operation at a dilution rate of 0.3 1/day. This phase continued until a steady state was achieved, defined as three full volume renewals with constant biomass concentration for at least three consecutive days. Evaporative losses were compensated

daily with deionized water. Finally, the harvested biomass and supernatant were separated via centrifugation and stored at -20°C for subsequent analysis.

2.4. Analytical Methods

Microalgal growth was monitored daily by measuring optical density at 750 nm (OD 750) and 680 nm (OD 680) using a UV-Vis spectrophotometer (Thermo Scientific Genesys 10S). The OD 680 was specifically recorded to reflect the maximum absorbance of the phtosynthetic pigments. To determine the dry weight biomass concentration (C_b, $g \cdot L^{-1}$), 50 mL aliquots of the culture were filtered through Macherey-Nagel glass fiber filters (MN 85/90) and dried at 80°C for 24 h. In semi-continuous mode, biomass productivity (P_b, $g \cdot L^{-1} \cdot d^{-1}$) was calculated using the following equation:

$$P_b = C_b \times D$$

where D represents the dilution rate (0.3 d^{-1}).

The maximum potential quantum yield of photosystem II (Fv /Fm) was determined using an AquaPen AP 100 fluorometer (Photon System Instruments, Czech Reublic). To ensure all reaction centers were open, microalgal samples were dark-adapted for 5 min prior to fluorescence measurements.

Liquid samples from the photobioreactor inlet and outlet were analyzed according to official analytical methods endorsed by the Spanish Ministry of Agriculture [8]. Nutrients: Phosphorus was determined via visible spectrophotometry using the phospho-vanado-molybdate complex, Nitrates (NO_3^-) were quantified spectrophotometrically (range: 220–275 nm), and ammonium ($NH_4^{+)}$ was determined using the Nessler reagent method. Chemical Oxygen Demand (COD): COD was measured using Hach-Lange kits (LCK-555 and LCI-400) followed by spectrophotometric measurement. Elemental and Ionic Composition The metal ion content of the recycled water was analyzed by an accredited laboratory using an inductively coupled plasma optical emission spectrometer (ICP-OES). Anion concentrations were quantitatively determined by ion chromatography (Metrohm 881 Compact IC pro).

2.5. Determination of Biomass Composition

Upon reaching a steady state, the biomass of *C. sorokiniana* and *S. obliquus* was harvested via centrifugation at 5,500 rpm (Rotanta 460, Hettich®, Poland). The resulting pellets were lyophilized to ensure stability before quantifying the total protein, lipid, and carbohydrate content.

Total protein was quantified using a modified Lowry method as proposed by Herbert et al. (1971) and Lowry et al. (1951) [17], [18]. Approximately 5 mg of freeze-dried biomass were digested in 1 N NaOH at 95°C for 1 h, following centrifugation at 4,000 rpm for 5 min. The supernatant was reacted sequentially with an alkaline copper reagent and the Folin–Ciocalteu reagent. After incubating for 30 minutes at room temperature, the absorbance was determined at 750 nm. Protein concentrations were measured using a standard calibration curve of bovine serum albumin (BSA). Total lipids were extracted and determined gravimetrically following the Folch method (1957) [19]. Lipids were isolated using a chloroform/methanol solvent mixture (2/1 v/v), and the final content was calculated based on the weight of the dried lipid extract. Carbohydrate concentration was measured using the phenol-sulfuric acid method, as described by Dubois et al. [20]. Briefly, 10 mg of microalgal powder were suspended in 10 mL of deionized water and subjected to sonication. A 50 µL aliquot of the sample was reacted with 150 µL of concentrated sulfuric acid and 30 µL of 5% (w/v) phenol. After color development, the optical density was measured at 490 nm. Carbohydrate concentrations were calculated as glucose equivalents.

2.6. Assessment of Biostimulant Activity

Microalgal biomass harvested at steady state underwent cell wall disruption using a UP 400 S ultrasonic processor (Hielscher Ultrasonics, Germany), following the protocol written by Morillas-España et al. (2022b) [12]. The resulting crude extracts were diluted with distilled water to the specific concentrations required for each bioassay.

The gibberellin-like activity was evaluated using the germination index (GI) of watercress (Lepidium sativum L.) seeds. Extracts were tested at concentrations of 0.1 and 0.5 $g \cdot L^{-1}$, with distilled water as the negative control .25 seeds were placed on Whatman No. 5 filter paper in sterile Petri dishes for each treatment, and then treated with 2 mL of the test solution. After three days of incubation at 24 °C in complete darkness, the GI (%) was calculated as follows:

$$GI(\%) = \left(\frac{N \cdot L}{N_c \cdot L_c} \right) \times 100$$

Where N and L represent the number of germinated seeds and average root length of the treated samples, respectively, while Nc and Lc refer to the distilled water control.

The auxin-like effect was assessed using soybean (Glycine max L.) hypocotyls. Seeds were sown in moistened perlite at 27°C with a 12:12 h photoperiod. After 7 days, seedlings were excised 3 cm below the cotyledon and placed in vials containing 20 mL of either microalgal extract 0.5 or 2.0 g L^{-1}. distilled water (negative control), or Indole-3-butyric acid (IBA, positive control). After an additional 7 days of incubation, the number of adventitious roots (length >1 mm) per hypocotyl was recorded. Results were expressed as a percentage of variation relative to the negative control.

Cytokinin-like activity was evaluated using cucumber (*Cucumis sativus L.*) cotyledons. Seeds were germinated for 4 days, after which uniform cotyledons were collected, weighed (10 units per dish), and transferred to Petri dishes containing 3 mL of test solution. Treatments included microalgal extracts (0.5 or 2.0 g L^{-1}) distilled water, and kinetin (5 mg L-1) as a positive control. After 3 days of incubation at 25°C in darkness, the increase in fresh weight was measured and compared against a kinetin calibration curve.

To determine chlorophyll retention, leaf segments (10 mm in length) were excised and incubated under controlled conditions (25 °C and 65% relative humidity) for a total period of 10 days. The samples were placed in vials containing 10 mL of treatment solution, including distilled water as a control, microalgal extracts at concentrations of 0.5 and 2.0 g L^{-1}, and 6-benzylaminopurine (BAP) as a positive control. After 4 days of incubation, chlorophyll was extracted by immersing the leaf segments in 80% methanol and heating at 80 °C for 10 minutes. The absorbance of the extracts was then measured at 645 nm, and chlorophyll retention was quantified by comparison with the control treatment.

2.8. Statistical Analysis

All experiments were performed in at least triplicate, and results are expressed as the mean ± standard deviation (SD). Data were subjected to a one-way analysis of variance (ANOVA) to

234 determine significant differences between treatments. When the null hypothesis was rejected
235 ($p<0.05$), Fisher's Least Significant Difference (LSD) test was applied for post-hoc multiple
236 steady-state comparisons. Statistical analyses were conducted using Statgraphics Centurion
237 XIX software. In all figures, error bars represent the standard deviation, and lowercase letters
238 are used to denote groups with no statistically significant differences at a 95% confidence
239 level.

240 **3. Results and Discussion**

241 **3.1. Biomass Productivity and physiological estate**

242 The robustness and growth efficiency of the selected microalgal strains were evaluated using
243 untreated wastewater as a culture medium. Steady-state biomass productivity profiles (Fig. 3A)
244 demonstrated that maximal biomass concentrations in raw wastewater were comparable to
245 those achieved in freshwater controls. Specifically, *S. obliquus* and *C. sorokiniana* reached
246 biomass concentrations of 1.2 ± 0,22 $g \cdot L^{-1}$ and 1.16 ± 0,00 $g \cdot L^{-1}$**,** respectively, corresponding
247 to volumetric productivities 0.36 ± 0,06 $g \cdot L^{-1} \cdot day^{-1}$ and 0.34 ± 0.00 $g \cdot L^{-1} \cdot day^{-1}$ (Fig. 3B). The
248 lack of significant differences between the two strains reflects the stability and uniformity of
249 the imposed environmental conditions. These results align with established benchmarks in the
250 literature. For instance, Morales et al.[16] evaluated several strains, including *S. obliquus*, in
251 non-sterilized primary wastewater under Nordic conditions and reported a robust biomass
252 concentration of 1.36 ± 0.27 $g \cdot L^{-}$. Furthermore, previous studies have highlighted the
253 adaptability of *C. sorokiniana* to autotrophic growth in raw wastewater, even at lower
254 concentrations 0.169 $g \cdot L^{-1}$ [5] which highlights its adaptability to wastewater-based systems.
255 Similarly, [12] reported a biomass concentration of 1.2 $g \cdot L^{-1}$ and a productivity of 0.38
256 $g \cdot L^{-1} \cdot day^{-1}$ for *Chlorella vulgaris* operated at a dilution rate of 0.3 day^{-1}, confirming the
257 suitability of *Chlorella* species for semi-continuous cultivation. The ability of untreated
258 wastewater to sustain productivities equivalent to synthetic media underscores its potential as
259 a nutrient-rich substrate for large-scale cultivation. This high adaptability in primary wastewater
260 is further supported by Amaya-Santos et al.[21] ,who observed similar resilience in *Chlorella*
261 strains.

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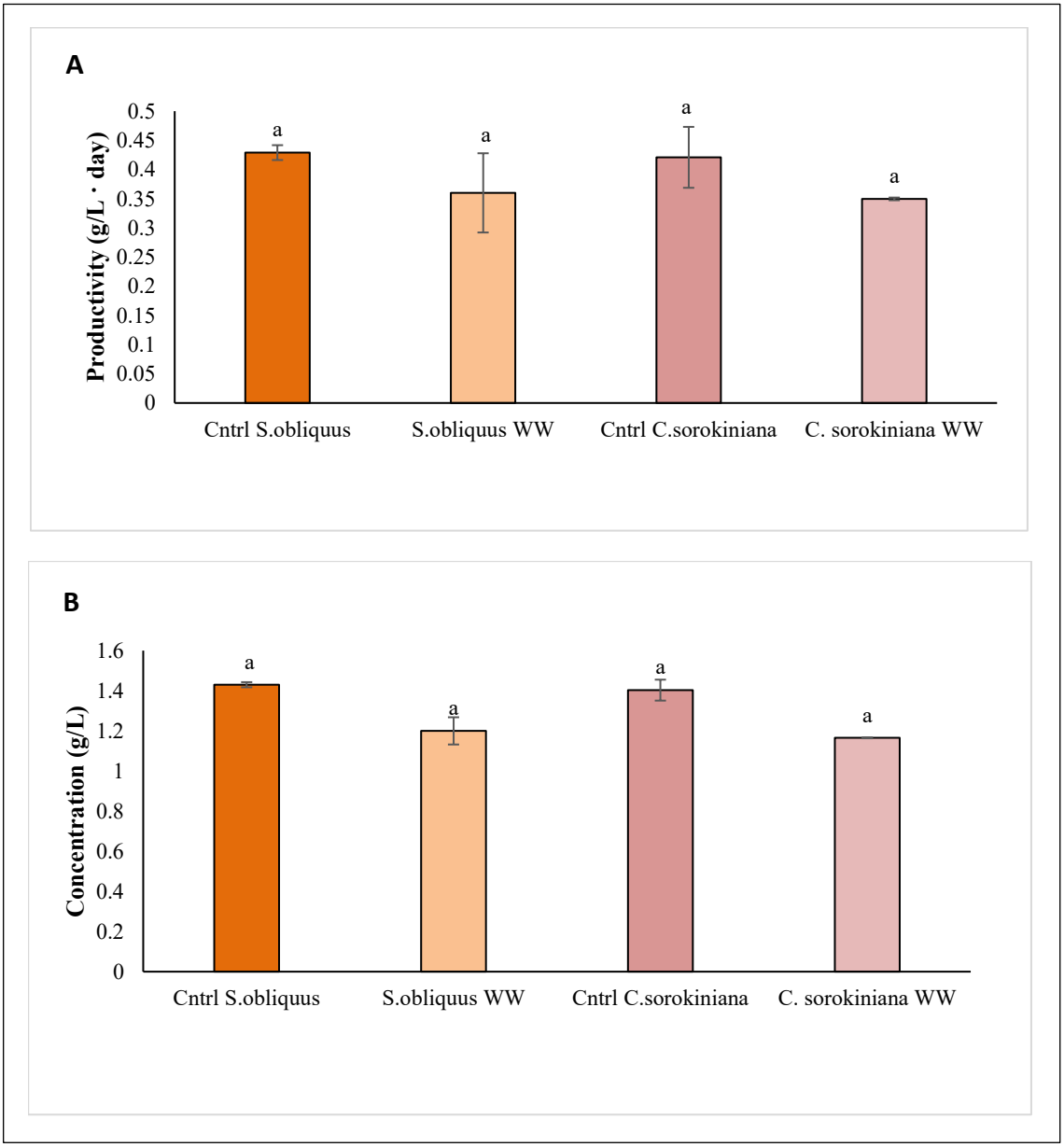


Fig. 3: (A) Biomass productivity during the semi-continuous production and **(B)** Biomass concentration at the end of the semi-continuous phase. Values are expressed as mean ± SD (n = 3). Means followed by different uppercase letters are significantly different according to one-way ANOVA followed by Fisher's LSD test at $p < 0.05$.

Regarding the photosynthetic performance it was assessed via the maximal quantum yield of Photosystem II (Fv/Fm), which provides a non-invasive measure of the physiological health of the cultures (Fig. 4) Since the Fv/Fm ratio is highly sensitive to abiotic and biotic stressors, it serves as a reliable indicator of the microalgal response to the wastewater environment [15]. Optimal Fv/Fm values for healthy microalgae typically range between 0.6 and 0.7 [22]. In this

study, steady-state Fv/Fm values range between 0.61 to 0.70 ($p < 0.05$), suggesting excellent photon availability and adequate nutrient supply under the tested conditions. These elevated values were consistent with the high biomass productivities observed, indicating that neither the wastewater nor the presence of indigenous microorganisms induced significant photosynthetic inhibition. Both strains demonstrated exceptional acclimation to raw primary wastewater, which fully met their metabolic requirements. The fact that the medium supported high productivity without the need for sterilization to eliminate rotifers or bacterial consortia is a significant finding. This aligns with Jebali and al. (2015)[23] and represents a substantial advantage for industrial-scale production by reducing operational costs without compromising system performance.

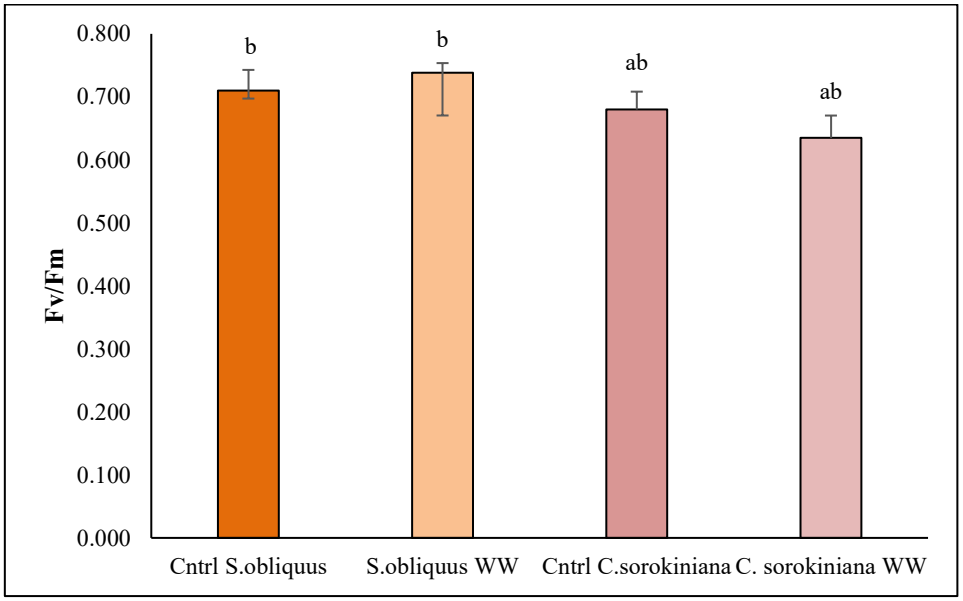


Fig. 4: Variation in Yield of photosystem II Fv/Fm values at steady state. Values are expressed as mean ± SD (n = 3). Means followed by different uppercase letters are significantly different according to one-way ANOVA followed by Fisher's LSD test at $p < 0.05$.

3.3. Phycoremediation of Primary Wastewater

A primary objective of this study was to evaluate the nutrient recovery potential of *S. obliquus* and *C. sorokiniana*. The initial ammonium (NH4+) concentration in the wastewater was 145.37 mg. L^{-1}. After treatment, *S. obliquus* achieved a remarkable reduction to 0.11 mg. L-1 (99.91% removal), while *C. sorokiniana* reached 1.05 mg. L^{-1} (99.27% removal) (Tab. 1). This near-total depletion suggests efficient assimilation for cell biosynthesis [1]. The slightly superior performance of *S. obliquus* aligns with Osundeko et al. (2013) [24] , who noted its high

tolerance and rapid assimilation kinetics under high nutrient loads. This efficiency is attributed to the directly assimilable nature of NH4+ via the GS-GOGAT pathway, which by passes the energy-intensive reduction step required for nitrates (NO_3^-) [25]. Furthermore, initial NO_3^- levels (1.5 mg. L^{-1}) were reduced to 0 mg. L^{-1} (100% removal) for both strains, surpassing results from Chen et al et al. (2018) [26],where residual nitrates indicated nitrification by bacteria that was not fully compensated by algal uptake. Regarding phosphorus, both species achieved over 81% removal (from 9.77 to 1.83 mg. L^{-1}. This performance is consistent with the "luxury uptake" mechanism, where microalgae store excess phosphorus as intracellular polyphosphates in specialized vacuoles [27]. This process, combined with initial biosorption via functional groups (carboxyl, hydroxyl, amine) on the cell wall, ensured compliance with EU Directive 91/271/EEC (minimum 80% P-reduction).

Regarding organic matter, the initial Chemical Oxygen Demand (COD) of 220,6 ±3.5 mg L^{-1} was significantly reduced, with removal rates of 58% for *C. sorokiniana* and 55.91% for *S. obliquus* (Tab.1). While microalgae contribute to COD reduction, the presence of associated heterotrophic bacteria likely played a synergistic role in oxidizing organic matter [23]. The final effluent remained well below the regulatory limit of 125 mg O_2 L^{-1} (Directive 98/15/EC), matching the quality standards for irrigation water (~92 mg L^{-1}). This enhanced removal is related to the combined action of photosynthetic oxygen production by microalgae and aerobic degradation pathways carried out by bacteria, which together improve the mineralization of organic compounds. Furthermore, part of the organic carbon is assimilated into the growing biomass, contributing to simultaneous pollutant removal and resource recovery. These results confirm the robustness of microalgae–bacteria consortia for treating organic-rich wastewaters and highlight their potential for integrated water treatment and biomass valorization within a circular economy framework.

Regarding heavy metals, although initial heavy metal concentrations were low (ppb level), significant reductions were observed, confirming active biosorption and bioaccumulation mechanisms. Iron (Fe): Reductions of 80.01% (*C. sorokiniana*) and 55.95% (*S. obliquus*) were recorded. Toxic Metals: *C. sorokiniana* showed higher efficiency in removing, Ni (38.25%), and Cu (43.58%) compared to *S. obliquus* (Tab.1). These results are consistent with (Sarma et al, 2024) [28], highlighting *Chlorophyta* as ideal candidates for phycoremediation. All residual metal concentrations remained below FAO (2019) thresholds for agricultural reuse. This removal performance is attributed to the affinity of microalgal cell walls for metal ions,

mediated by functional groups such as hydroxyl, carboxyl, and amine groups that facilitate adsorption processes. Additionally, intracellular uptake and sequestration mechanisms likely contributed to the stabilization and detoxification of metals within the biomass. The differences observed between strains suggest species-specific interactions with metal ions, influenced by cell wall composition and metabolic activity.

Ionic dynamics were also monitored to assess the potential risks of soil salinity associated with the reuse of treated effluent. Initial concentrations of chloride (473.4 mg L^{-1}), sodium (250.4 mg L^{-1}), and sulfates (121 mg L^{-1}) remained within FAO/WHO [29] safety limits after treatment. The observed fluctuations in ionic composition are likely attributed to adsorption–desorption processes and cation exchange mechanisms occurring at the microalgal cell surface [30]. Despite a moderate electrical conductivity, the treated effluent can be classified as suitable for irrigation with appropriate management practices, providing a stabilized source of nutrients for crop development [31]. These results indicate that microalgae-based treatment does not significantly increase salinity risks and may contribute to maintaining a balanced ionic profile in the reclaimed water. Moreover, the partial uptake and transformation of ions by the microalgal biomass can help buffer sudden variations in water composition

373 Table1: The removal efficiency of some nutrients (mg/L), heavy metals (µg/L), minerals, and
374 COD (mg/L) from wastewater using *C. sorokiniana*, and *S. obliquus*, along with the
375 consideration of standard error. Means sharing different letters are significantly different, as
376 determined at $p < 0.05$.

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Parameters	Initial concentrations	After treatment			
		C. sorokiniana	% of removal	*S. obliquus*	% of removal
pH	8±0.21 a	7.93 ±0.05 a	-	7.76±0.06 a	-
CE	3.28±0.21 a	2.58±0.24 b	-	1.52±0,.31 c	-
NO_3^- (mg/L)	1.53±0,0.06 a	0±0.00 b	100	0±0.00 b	100
NH_4^+ (mg/L)	145.37 ± 0.3 a	1,05±0.04 b	99.28	0.11±0.01 c	99.92
$PO4^{3-}$ (mg/L)	9.76±0.35 a	1.83±0.00 b	81.25	1.83±0.00 b	81.25
Fe^{2+} (µg/L)	34.32±0.21 a	6.86±0.25 c	80.01	14.09±0.35 b	58.95
Cr (µg/L)	0.374±0.30 a	0.262±0.00 b	29.94	0.260±0.00 b	30.48
Ni^{2+}(µg/L)	2.17±0.20 a	1.34±0.01 b	38.25	1.36±0.00 b	37.33
Cu^{2+} (µg /L)	4.13±0.22 a	2.33±0.29 b	43.58	3.35±0.6 ab	18.89
Cl^-	474.1±4.68 b	531.3±11.33 a	-	516.23±2.04 a	-
SO_4^{2-}	123.3±2.06 b	140.68±0.88 a	-	126,5±1.51 b	-
Ca^{2+} (mg/L)	83.8±0.65 a	70.97±0.87 c	-	81.13±1.13 b	-
Mg^{2+}(mg/L)	33.21±1.07 b	39.6±0.88 a	-	37.37±0.6 b	-

Na^+ (mg/L)	251.66±2.63 c	303.08±1.05 a	-	284.23±5.29 b	-
K^+(mg/L)	32.00±1 b	38,33±0.57a	-	28.67±1.15 c	-
F^-(mg/L)	1.9±0.11 a	1.9±0.11 a	-	0±0.00 b	100
Br^-(mg/L)	0±0.00	0±0.00	-	0±0.00	-
COD (mg/L)	220.6±3,05 a	90.83±0,58 c	58.83	97.66±2.08 b	55.73

Principal Component Analysis (PCA) (Fig. 5) accounted for 94.8% of the total variance, providing a robust representation of the system behaviour. The first principal component (Dim 1, 84%) clearly separated raw wastewater from treated effluents, defining a strong pollution gradient driven by high loadings of NH_4^+, COD, PO_4^{3-}, and NO_3^-. The clear displacement of both microalgal treatments (*C. sorokiniana* and *S. obliquus)* away from the influent along this axis reflects the substantial reduction of these contaminants and confirms the high remediation efficiency achieved under the studied conditions.

The second principal component (Dim 2, 10.8%) provided further insight into species-specific behaviour by discriminating between the two microalgal systems. *C. sorokiniana* was associated with higher pH and electrical conductivity, indicating a stronger impact on the physicochemical environment, likely linked to more intense photosynthetic activity and CO_2 uptake. In contrast, *S.obliquus* was more closely aligned with metal-related variables such as Fe^{2+} and Cu^{2+}, suggesting differences in metal interaction mechanisms, including adsorption or intracellular accumulation. The spatial distribution of variables and samples in the PCA plot highlights the complementary roles of both species, not only in nutrient removal but also in shaping the overall water chemistry. This multivariate analysis therefore reinforces the suitability of both strains for wastewater treatment, while also revealing distinct functional traits that could be exploited depending on the target contaminants and treatment objectives.

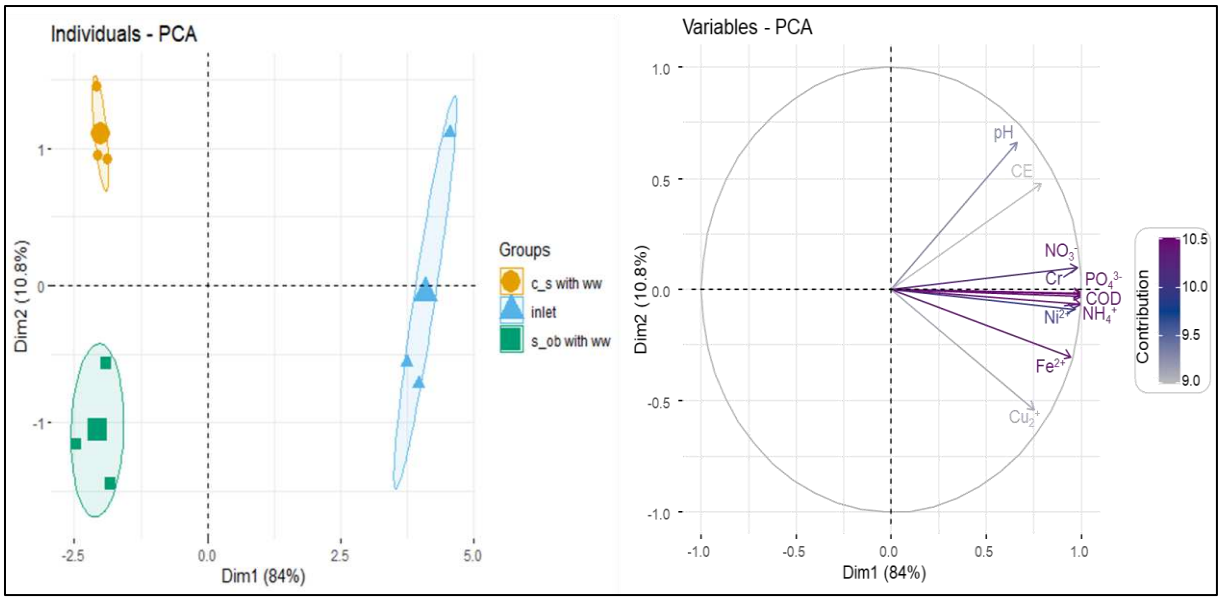


Fig.5. Principal Component Analysis (PCA) of physicochemical parameters highlighting the effect of *C. sorokiniana* and *S. obliquus* on wastewater treatment.

3.4. Biochemical Composition of the Produced Biomass

The biochemical composition of the biomass at steady state was assessed to determine its potential for utilization (Fig. 6). The results highlight distinct metabolic strategies between *C.sorokiniana* and *S. obliquus* when cultivated in primary treated wastewater (PWW) compared to the synthetic control medium. *C. sorokiniana* maintained a protein-rich profile, with content reaching 46.39% DW in the control and 43.40% DW in wastewater. This high protein fraction reflects intense metabolic and photosynthetic activity, a characteristic of fast-growing autotrophic microalgae [32]. The slight decrease in wastewater suggests a minor metabolic shift, yet the profile remains highly suitable for agricultural applications. This nitrogen-rich composition makes *C. sorokiniana* an excellent candidate for biostimulant production. Its protein fraction is rich in amino acids like tryptophan and arginine, which serve as precursors for essential phytohormones such as auxins and polyamines. These compounds are instrumental in stimulating plant growth, flowering, and abiotic stress tolerance [33]. This protein dominance was accompanied by a moderate increase in carbohydrates (21.62% in PWW vs. 15.03% in control) and a significant decrease in lipids (11.45% in PWW vs. 16.47% in control), indicating a carbon redistribution favoring structural and functional molecules over energy storage.

In contrast, *S. obliquus* exhibited a composition dominated by carbon-rich compounds. Carbohydrate levels remained high (39.07% in PWW and 41.89% in control), while lipid content decreased from 18.29% in the control to 12.4% in wastewater (Fig 6). This metabolic

orientation toward polysaccharides and lipids is a typical response to specific nutrient balances, where cells redirect carbon flux toward reserve compounds to maintain energy equilibrium [34]. These results are consistent with [35] , who reported protein dominance in *Chlorella* species versus a higher sugar content in *Tetradesmus* (*Scenedesmus*) *obliquus*. The robust morphology of *Scenedesmus*, characterized by a thick cell wall, further supports the accumulation of structural polysaccharides [23]. Additionally, high carbohydrate levels may serve a protective role against heavy metal traces in wastewater, as wall-associated polysaccharides can complex metal ions, limiting their intracellular toxicity [36].

Overall, the biochemical profiles observed align with literature ranges for wastewater-grown microalgae: proteins (20–50%), carbohydrates (15–45%), and lipids (8–30%) [35]. The results confirm that primary wastewater is an effective nutrient medium capable of supporting growth while inducing the metabolic shifts necessary for targeted applications. The protein-rich biomass of *C. sorokiniana* is highly promising for the production of high-value biostimulants and organic fertilizers. The carbohydrate and lipid-rich biomass of *S. obliquus* demonstrates strong potential for bioenergy applications, including bioethanol and biodiesel production [37].

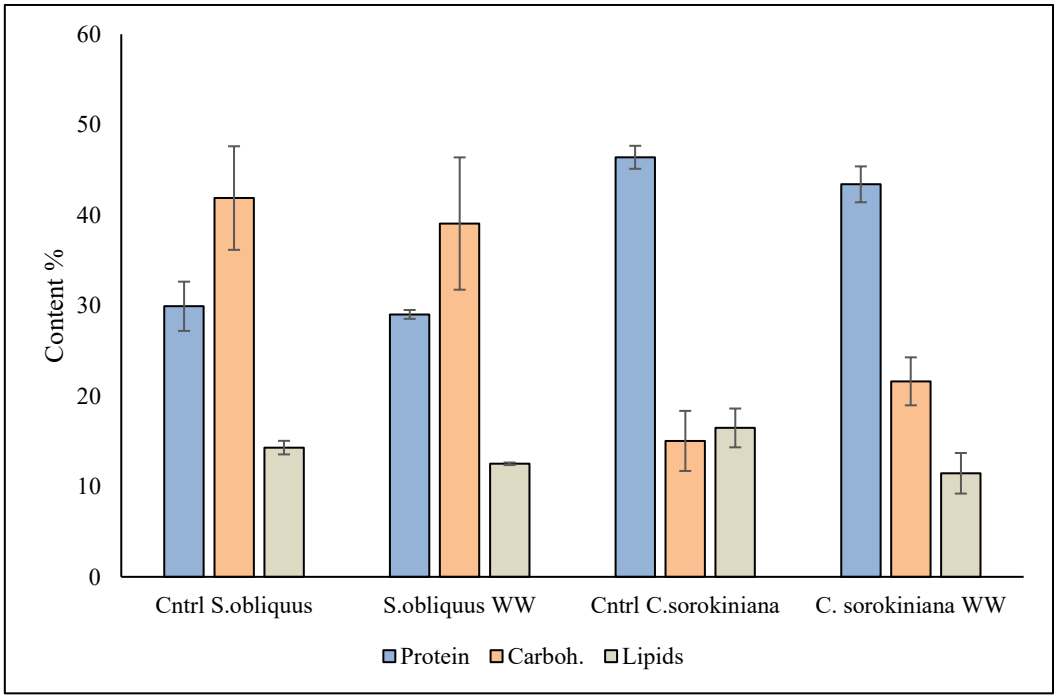


Fig. 6: Biochemical characterization of the algal biomass at steady state for the different dilution rates applied. Values are expressed as mean ± SD (n = 3). Means followed by different uppercase letters are significantly different according to one-way ANOVA followed by Fisher's LSD test at $p < 0.05$.

3.5. Biostimulant Activity of Microalgal Extracts

The production of agricultural biostimulants from microalgal biomass is a burgeoning field, offering sustainable alternatives to synthetic inputs by enhancing crop yields and nutrient use efficiency [12]. In this study, the biostimulant potential of *C. sorokiniana* and *S. obliquus* was characterized through four distinct bioassays to identify hormone-like activities.

Seed germination is a critical phase sensitive to both phytotoxicity and hormonal signaling. The germination index (GI) results (Fig. 7) showed that all treatments exceeded the 80% threshold, confirming the absence of phytotoxicity in extracts derived from wastewater-grown biomass [21]. The strongest stimulation was observed with *C. sorokiniana* (WW) at 0.5 g L^{-1}, yielding a GI of 135%. This performance is comparable to the positive control GA3 at 3 mg L^{-1}, GI 141%, suggesting a significant gibberellin-like effect. Gibberellins are essential for breaking seed dormancy and mobilizing nutrient reserves [38]. The superior response of *C. sorokiniana* compared to *S. obliquus* highlights a strain-dependent bioactive profile, consistent with previous reports indicating that *Chlorella* species are particularly effective at initiating early germination processes [21].

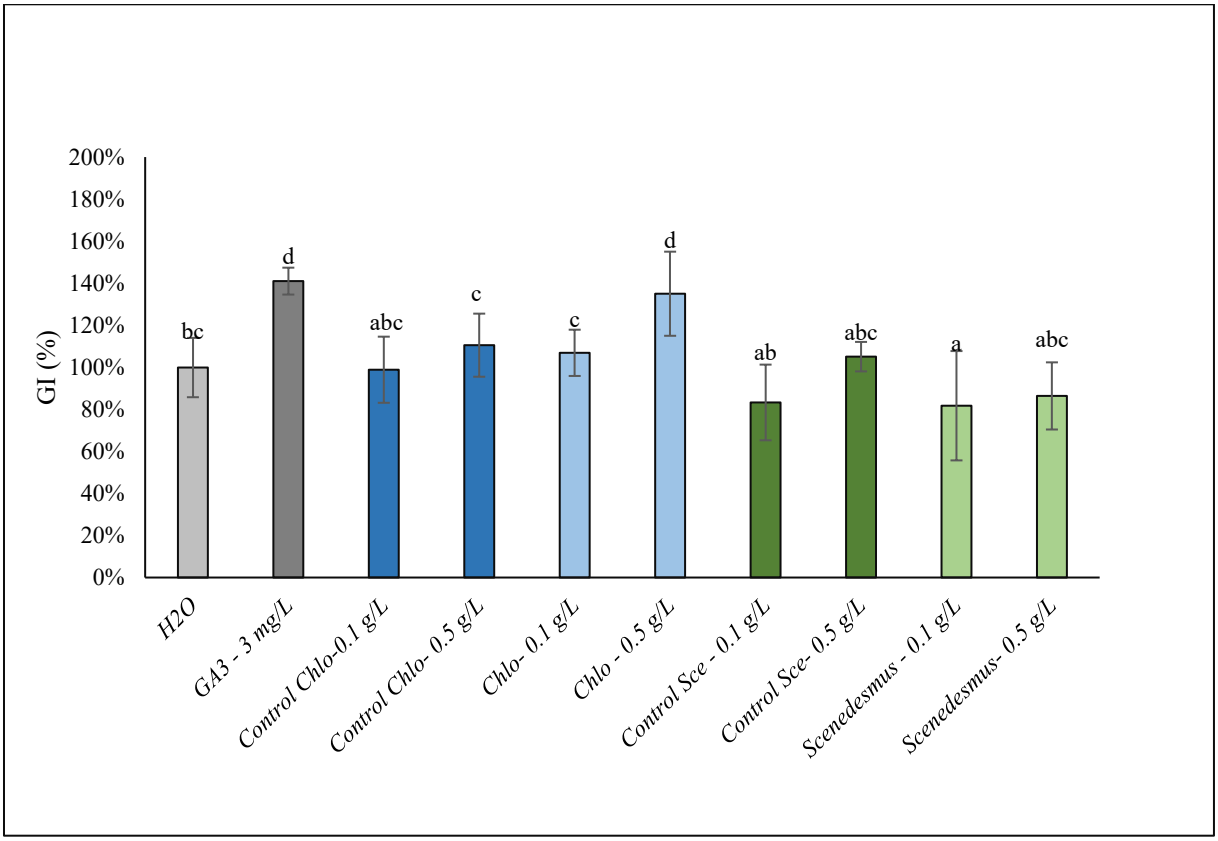


Fig. 7: Effect of the microalgal extracts on the germination index of watercress seeds. Values represent the percentage of variation with respect to the control (distilled water). Values are expressed as mean ± SD (n = 3). Means followed by different uppercase letters are significantly different according to one-way ANOVA followed by Fisher's LSD test at $p < 0.05$.

Auxins are the primary regulators of root initiation and architecture. As shown in Figure 8, all microalgal extracts significantly enhanced the formation of adventitious roots compared to the negative control ($p < 0.05$), demonstrating a clear auxin-like activity. The most pronounced effects were observed at a concentration of 2 g L^{-1}, where root formation reached 226% for *S. obliquus* and 218% for *C. sorokiniana*. These strong responses are consistent with the high protein and amino acid content identified in the biomass, particularly tryptophan, a known precursor of indole-3-acetic acid (IAA), the main endogenous auxin in plants. Similar auxin-like effects have been reported for *S. obliquus* cultivated in industrial effluents [39], [40], as well as for Scenedesmus-derived products applied at comparable concentrations [41]. The magnitude of the response observed in this study suggests that wastewater-grown microalgae can produce a complex mixture of bioactive compounds capable of stimulating root development. This enhanced rooting capacity is particularly relevant for improving plant establishment, anchorage, and nutrient uptake efficiency, especially under suboptimal growth conditions.

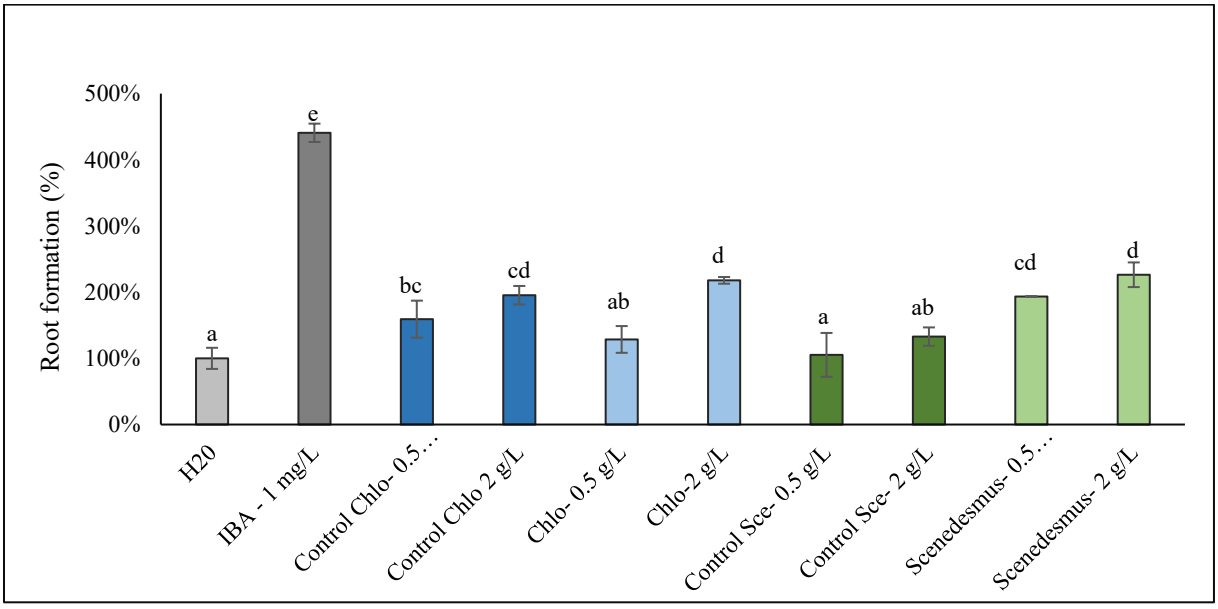


Fig. 8: Effect of the microalgal extracts on the formation of adventitious roots. Values represent the percentage of variation with respect to the control (distilled water). Values are expressed as

mean ± SD (n = 3). Means followed by different uppercase letters are significantly different according to one-way ANOVA followed by Fisher's LSD test at $p < 0.05$.

Cytokinins are key phytohormones that regulate cell division, promote tissue expansion, and delay leaf senescence by maintaining chlorophyll content and metabolic activity. To evaluate cytokinin-like activity, two complementary bioassays were performed, providing insight into both growth promotion and anti-senescence effects. In the cucumber cotyledon expansion assay (Figure 9), *C. sorokiniana* extracts at 2 g L^{-1} induced a strong growth response, with increases of 77% and 69%, surpassing the effect of the reference cytokinin (BAP at 1 mg L^{-1}). This result indicates a high concentration of cytokinin-like compounds or precursors in the biomass, likely associated with its protein-rich composition and active metabolic profile under wastewater conditions. The ability to exceed the synthetic control suggests that microalgal extracts may offer a complex mixture of bioactive molecules acting synergistically, rather than a single hormone analogue.

In contrast, *S. obliquus* grown in wastewater exhibited a comparatively lower effect in this assay, suggesting differences in metabolic allocation and bioactive compound production between species. This reduced response may indicate that its biochemical profile is less enriched in cytokinin-like substances or that its activity is directed toward other physiological pathways, such as root stimulation, as observed in the adventitious root assay. Additionally, variations in cell wall composition and extraction efficiency could influence the availability of active compounds. These results highlight a clear species-dependent functional specialization, where *C. sorokiniana* appears more suitable for applications targeting aerial plant growth and biomass accumulation, while *S. obliquus* may be more effective in promoting root development. Overall, the findings reinforce the importance of strain selection and cultivation conditions in tailoring microalgal biostimulants for specific agricultural applications.

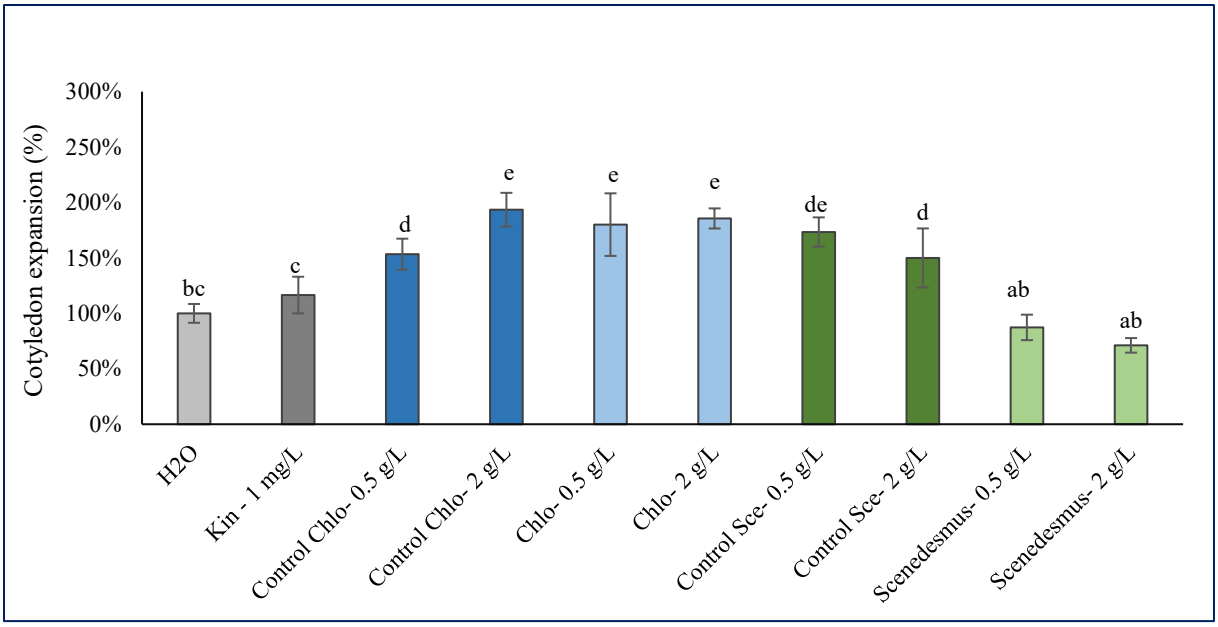
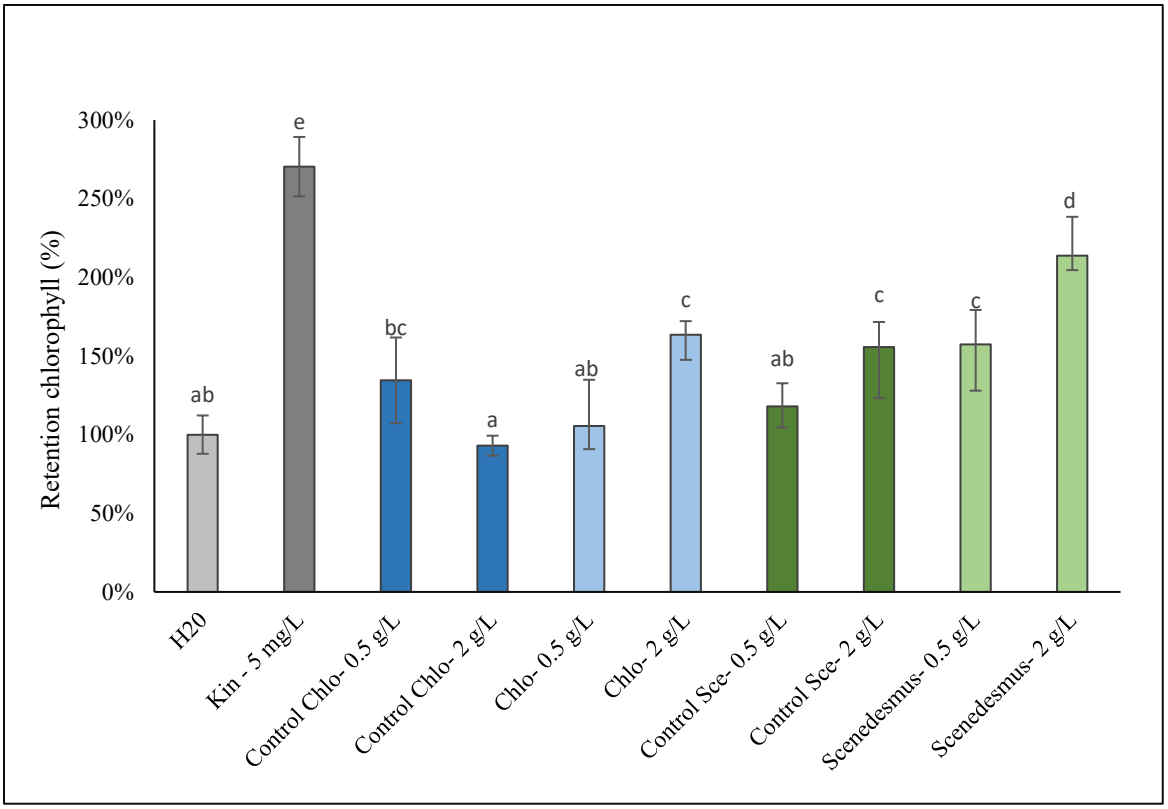


Fig. 9: Effect of the microalgal extracts on the weight of cucumber cotyledons. Values represent the percentage of variation with respect to the control (distilled water). Values are expressed as mean ± SD (n = 3). Means followed by different uppercase letters are significantly different according to one-way ANOVA followed by Fisher's LSD test at $p < 0.05$.

Wheat chlorophyll retention (Fig. 10) demonstrated that all microalgal extracts significantly delayed chlorophyll degradation, confirming their cytokinin-like, anti-senescence activity. The highest level of stability was observed for S. obliquus (WW) at 2 g L^{-1}, reaching 214% retention, a value comparable to the BAP control. This strong effect indicates an efficient preservation of the photosynthetic apparatus, likely associated with bioactive compounds that inhibit chlorophyll breakdown and maintain cellular functionality. Notably, these results show that even if a strain exhibits a lower performance in cell expansion assays, it may still possess a high capacity to delay senescence. This highlights the presence of functionally distinct cytokinin-like molecules or related metabolites, reinforcing the potential of *S. obliquus* extracts as effective anti-senescence agents in agricultural applications [12].



520

521 **Fig.10:** Effect of the microalgal extracts on the chlorophyll content of the detached wheat
522 leaves. Values represent the percentage of variation in the ABS 645 with respect to the control
523 (distilled water). Values are expressed as mean ± SD (n = 3). Means followed by different
524 uppercase letters are significantly different according to one-way ANOVA followed by Fisher's
525 LSD test at $p < 0.05$.

526 The four bioassays collectively demonstrate that wastewater is not only a suitable medium for
527 microalgal biomass production but also an effective driver for the synthesis of bioactive
528 compounds with biostimulant properties. The intensity and nature of the observed effects are
529 strongly influenced by the interaction between the microalgal strain and the cultivation
530 conditions, highlighting the importance of process optimization. In particular, *C. sorokiniana*
531 emerged as a highly versatile biostimulant, showing consistent and superior performance across
532 germination, root induction, and cotyledon expansion assays. Although these results are
533 promising, translating laboratory-scale findings into practical agricultural applications will
534 require further validation under greenhouse and field conditions to assess their effectiveness
535 under real agronomic scenarios. Nevertheless, the integration of wastewater treatment with the
536 production of high-value biostimulants represents a compelling circular economy approach,

simultaneously addressing environmental remediation and the development of sustainable inputs for agriculture.

Conclusion

The findings of this study show that effective wastewater recycling and reuse can be achieved through a dual-purpose bioremediation strategy based on the use of *C. sorokiniana* and *S. obliquus*. Both species showed outstanding nutrient removal performance, with ammonium removal efficiencies exceeding 99% (from 145.37 mg L^{-1} to values as low as 0.11 mg L^{-1}) and phosphorus reductions above 81%. These results confirm the strong potential of microalgae-based systems for advanced wastewater treatment and nutrient recovery. In addition to their remediation capacity, the produced biomass exhibited significant value for agricultural applications. In particular, *C. sorokiniana* demonstrated a high biostimulant potential, increasing the germination index up to 135% and enhancing cell expansion by 77% compared to the control. These findings highlight its capacity to produce bioactive compounds with hormone-like activity, reinforcing its suitability for integration into sustainable agricultural practices and industrial-scale bioprocesses.

Although these results were validated at laboratory scale using controlled photobioreactors, further work is required to assess system performance under real outdoor conditions. The transition to pilot-scale systems will be essential to evaluate process robustness under variable environmental factors. In this context, future studies should also consider the integration of an additional tertiary treatment step to achieve Category A water quality, enabling the safe reuse of treated effluents for irrigation purposes. Finally, to support large-scale implementation, the technical feasibility demonstrated in this work must be complemented by comprehensive economic assessments and life cycle analyses. Such integrated evaluation will be critical to position microalgae-based wastewater treatment as a viable and scalable solution within the framework of a sustainable circular economy.

Author contributions

Hidaya Elgazaiel: Formal Analysis, Methodology, Conceptualization, Investigation, Data Curation, Writing - Original Draft, Writing review and editing.

Cintia Gómez-Serrano: Validation, Visualization.

Neila Hkiri: SEM scanning**,** Visualization.

Lucía González Fernández-Amela: Methodology, Investigation, Validation, Writing - Original Draft.

Francisco Gabriel Acien Fernández: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing review and editing, Supervision, Project administration.

Nerda Asses: Conceptualization, Visualization, Writing - Original Draft, Writing review and editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Achnowlegements

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